REMARKS

Claim Amendments

Claims 1–10, 14–21, and 25 are pending. Claims 8–10, 14, 22, and 25 are amended. Claims 1-7 and 15-21 are withdrawn. Claims 11–13, 23, and 24 are cancelled herein without prejudice or disclaimer. Applicant reserves the right to file continuation or divisional patent application(s) drawn to cancelled subject matter.

Support for the claim amendments can be found throughout the specification and in the claims as originally filed.[1] Applicant respectfully requests entry of the above amendment and submit that the amendments do not constitute new matter.

Claim Rejection — 35 U.S.C. § 112, first paragraph (written description)

Claims 8–14 and 22–25 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement.

The Office Action contends that the specification does not provide written description for the genus of substances "capable of acting on the NP receptor GC-A to enhance cGMP production."[2] The Office Action acknowledges, however, that the specification describes the ANP and BNP peptides of SEQ ID NOs: 1–8, and that these peptides are species of the genus of "substances."[3]

As amended, the claims comprise the step of administering (or treating with) an atrial natriuretic peptide (ANP) or brain natriuretic peptide (BNP).

ANP and BNP are well known in the art.[4] ANP is secreted from the heart and plays an important role in water-electroyte metabolism and blood pressure regulation.[5] The specification discloses the amino acid sequences of a variety of ANP species including human ANP (*e.g.*, SEQ

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[1] *See, e.g.*, Specification, ¶¶ 2, 5, 13, and 14. The specification citations refer to the paragraph numbers in Pub No. US 2007/0270337, the publication of the instant application.

[2] *See* Office Action, pages 2 and 3.

[3] *See id.* at pages 3 and 4.

[4] *See* Specification, ¶¶ 13-15 (*e.g.*, teaching that ANP and BNP are specific ligands for guanylyl cyclase A (GC-A)); *see also* Koller and Goeddel "Molecular biology of the natriuretic peptides and their receptors." Circulation (1992) 86: 1086-1088. (**Exhibit A**) . Prior to the instant invention, however, ANP and BNP were not known to be useful in treating Th1-mediated immune diseases or in regulating the Th1/Th2 balance in the immune system.

ID NO: 1), rat ANP (*e.g.*, SEQ ID NO: 2), and frog ANP (SEQ ID NO: 5), as well as a useful fragment of human ANP (SEQ ID NO: 3).[6]

BNP is a related cardiac hormone with similar diuretic, natriuretic, and vasodilatory activities. The specification also discloses various BNP amino acid sequences including human BNP (SEQ ID NO: 4), pig BNP (SEQ ID NO: 6), rat BNP (SEQ ID NO: 7), and chicken BNP (SEQ ID NO: 8).[7] Based on ANP and BNP amino acid sequence information, those skilled in the art may make and use modified ANP and BNP sequences by techniques known in the art such as deletion, substitution, addition and/or insertion of amino acid residues in the ANP or BNP sequences.[8] Moreover, ANP and BNP may be isolated in pure form from natural sources, chemically synthesized, or recombinantly produced.[9]

As discussed above, at the time the instant application was filed, ANP and BNP were well characterized natriuretic peptides. As such, the specification reasonably conveys to one of skill in the art that Applicant was in possession of the claimed invention, at the time the application was filed. Accordingly, Applicant respectfully requests withdrawal of the written description rejection.

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[6] *See* Specification, ¶¶ 19 and 20; *see also* Sequence Listing.

[7] *See* Specification, ¶¶ 21 and 22; *see also* Sequence Listing.

[8] *See* Specification, ¶ 22.

[9] *See id.*

Claim Rejection — 35 U.S.C. § 112, first paragraph (enablement)

Claims 8–14 and 22–25 stand rejected under 35 U.S.C. § 112, first paragraph, because the specification, while being enabled for *decreasing graft rejection following transplantation comprising administering an effective amount of structurally and functionally characterized natriuretic peptides of SEQ ID NOs: 1–8*, allegedly does not reasonably provide enablement for *"treating" or preventing known symptoms related to undefined "Th1-mediated immune disease" with unknown etiology.*

As amended, claim 8 is drawn to a method for treating a Th1-mediated immune disease comprising administering ANP or BNP, wherein said disease is a disease due to graft rejection following transplantation or multiple sclerosis. Claim 22, as amended, is drawn to a method for regulating the Th 1/Th2 balance in the immune system comprising treating dendritic cells with an ANP or BNP, thereby polarizing T cells toward Th2-promoting phenotype. As discussed below, the specification enables the full scope of these claims.

Dendritic Cell Polarize naïve T Cells into Th1 Helper Cells and Th2 Helper Cells

Dendritic cells are antigen-presenting cells that are activated after interaction with pathogens and various inflammation associated factors including cytokines.[10] After activation, these cells polarize naïve T-cell development into T helper 1 (Th1) or T helper 2 (Th2) cells.[11] Th1 and Th2 cells are distinguished by the cytokines they produce, the cytokines to which they respond, and the immune responses in which they are involved. Th1 cells produce pro-inflammatory cytokines (*e.g.*, IFN-γ, TNF-β, IL-2, and IL-12), while, in contrast, Th2 cells produce the cytokines (*e.g.*, IL-4, IL-5, IL-6, IL-10, and IL-13) that suppress the release of pro-inflammatory cytokines (*e.g.*, TNF-α).[12] Immune reactions are modulated by a balance of Th1 and Th2 helper cell activity.[13]

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[10] Sallusto and Lanzavecchia (2002) "The instructive role of dendritic cells on T-cell responses." Arthritis Research 4(Suppl. 3): S127–S132 (**Exhibit B**)

[11] Giordano, *et al.* (2006) "Nitric oxide and cGMP protein kinase (cGK) regulate dendritic-cell migration toward the lymph-node–directing chemokine CCL19." Blood **107**(4): 1537–1545 at 1537 (**Exhibit C**).

[12] BioCarta- Charting Pathways of Life (Dendritic cells in regulating Th1 and Th2 Development) website downloaded on December 2, 2009. (**Exhibit D**)

[13] Specification at ¶ 3; *See also* Sallusto and Lanzavecchia (2002) "The instructive role of dendritic cells on T-cell responses." Arthritis Research 4(Suppl. 3): S127–S132 (**Exhibit B**)

Th2 Helper Cells Inhibit Th1-mediated Immune Reactions

Th2 cells inhibit helper cell-mediated immune reactions, and thus, polarization of naïve T cells into Th2 cells are therapeutic.[14] For example, Vieira teaches that glatiramer acetate (Copolymer-1) is an effective therapeutic agent for multiple sclerosis. Copolymer-1 suppresses the induction of experimental autoimmune encephalomyelitis (EAE)[15] and reduces the relapse frequency in relapsing-remitting multiple sclerosis. Copolymer-1 acts on dendritic cells to lower their secretion of IL-12 and increase secretion of the anti-inflammatory cytokine IL-10. This change in the dendritic cells cytokine profile results in the polarization of naïve T cells into Th2 cells.[16] This increase in Th2 cell polarization leads to decreased inflammation and, consequently, the alleviation of the symptoms of multiple sclerosis. Moreover, Zhang reported that dendritic cells with high IL-10 and low IL-12 production administered to EAE mice resulted in the suppression of the symptoms of EAE.[17] Again, the administered dendritic cells increased the polarization of naïve T cells into Th2 cells which dampened the effects of Th1-mediated inflammation. Accordingly, the art demonstrates that agents which enhance Th2 polarization by dendritic cells are useful in treating multiple sclerosis because the Th2 cells suppress Th1 cytokine production and inflammation.[18]

ANP and BNP Activate Dendritic Cells to Polarize naïve T-Cells into Th2 Helper Cells

Applicants discovered that ANP and BNP bind guanylyl cyclase-A (GC-A)[19] expressed on dendritic cells. As taught in the specification, ANP and BNP activate dendritic cells inhibiting IL-12 and TNFα production and enhancing IL-10 production.[20] This activation drives T cell differentiation towards the Th2-type helper T cell type.[21] As discussed above, this leads to

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[14] Specification at ¶ 27.

[15] An art accepted model of multiple sclerosis.

[16] Vieira, et al. (2003) "Glatiramer Acetate (Copolymer-1, Copaxone) Promotes Th2 Cell Development and Increased IL-10 Production through Modulation of Dendritic Cells." The Journal of Immunology 170: 4483–4488 at 4484. (Exhibit E)

[17] Zhang, et al. (2002) "Mature bone marrow-derived dendritic cells polarize Th2 response and suppress experimental autoimmune encephalomyelitis." Multiple Sclerosis 8: 463–468 at 463 466. (Exhibit F)

[18] Specification at ¶ 17.

[19] Also known as natriuretic peptide receptor-A

[20] Specification ¶¶ 86–87, Figure 4.

[21] Specification at ¶ 29.

an inhibition of Th1-mediated immune reactions (*e.g.*, in multiple sclerosis).[22] Accordingly, the specification demonstrates that ANP or BNP may be used to treat a disease due to graft rejection following transplantation or multiple sclerosis because ANP and BNP act to suppress the pro-inflammatory immune response involving Th1-type helper T cells.[23]

The structure ANP and BNP are well-known in the art

As discussed above, the specification teaches two structurally and functionally characterized natriuretic peptides, ANP and BNP. Therefore, a person of skill in the art need not engage in undue experimentation to make and use the natriuretic peptides in the claimed methods.

In view of the foregoing, Applicant submits that the skilled artisan need not undertake undue experimentation to practice the claimed method and respectfully requests withdrawal of the enablement rejection.

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[22] Specification at ¶ 27.
[23] Specification at ¶ 29; *see also* Example 2.

CONCLUSION

Applicant respectfully submits that the pending claims are in condition for allowance, and such disposition is earnestly solicited. Should the Examiner believe that any issues remain after consideration of this Response, the Examiner is invited to contact the Applicant's undersigned representative to discuss and resolve such issues.
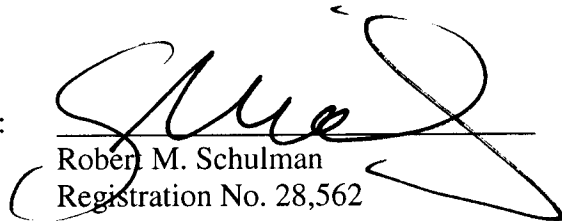
It is believed that no other fees are required for entry of these remarks, but should any fees be necessary, the Commissioner is authorized to charge such fees to **Deposit Account No. 50-0206**.

Respectfully submitted,

HUNTON & WILLIAMS LLP

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Exhibit A

Koller and Goeddel
"Molecular biology of the natriuretic peptides and their receptors."
Circulation (1992) 86: 1086-1088

⊞Research Advances Series

Focus on Molecular Biology

Molecular Biology of the Natriuretic Peptides and Their Receptors

Kerry J. Koller, PhD, and David V. Goeddel, PhD

Atrial natriuretic peptide (ANP) is the first described peptide in a family of hormones known to have important roles in the regulation of body fluid homeostasis.[1] It is secreted by atrial myocytes in response to increased intravascular volume. Once it is in the circulation, its effects are primarily on the kidney, vascular tissue, and adrenal gland, in which its actions lead to natriuresis, diuresis, and a decrease in intravascular volume and blood pressure.[2] The description of the potent diuretic and natriuretic properties of atrial extracts more than a decade ago was the first evidence that the heart could be an endocrine organ.[3] The subsequent isolation and characterization of this activity identified ANP as the first secreted cardiac hormone.[4-7] ANP's actions on both the cardiovascular system and the kidney as well as its role in pathophysiological states such as heart failure, hypertension, and renal disease have made it a molecule of great interest to both clinical and basic scientists.[1] The recent discovery of a family of structurally related hormones suggests that the physiological control of body fluid homeostasis is complex. This complexity is enhanced by the existence of at least three types of receptors specific for the natriuretic peptides. The aim of this review is to summarize the recent studies on the molecular biology of the natriuretic peptides and their receptors.

Natriuretic Peptides

After deBold et al's[3] initial description of natriuretic activity in rat atrial extracts, several groups isolated rat ANPs of various lengths but with common amino acid sequences.[4-8] Studies of the gene and messenger RNA (mRNA) encoding ANP demonstrated that the isolated peptides were various fragments of the naturally occurring hormone.[9-12] ANP is synthesized first as the 152-amino-acid prepro-ANP (Figure 1). Removal of the 24-amino-acid signal sequence (and the two carboxyl (C)-terminal arginine residues found in the rat, rabbit, and bovine but not human sequences) yields pro-ANP (1–126), the principal form of ANP stored in atrial granules.[13] The bioactive peptide is generated by proteolytic cleavage of pro-ANP into predominantly ANP (99–126) and the inactive ANP (1–99).[14] The active form of ANP (99–126) has a disulfide bridge between

cysteines 105 and 121, and all ANP analogues with natriuretic or diuretic activity share this common central ring structure.[8,15] Although pro-ANP is processed primarily to a 28-amino-acid hormone, as indicated in Figure 1, other forms of ANP have been identified. Urodilatin, first isolated from human urine and synthesized in the kidney,[16] represents atypical processing to an amino (N)-terminally extended peptide, ANP (95–126).[17] Alternatively processed forms of ANP have also been found in the testis (ANP [96–126])[18] and the brain (ANP [102–126] and ANP [103–126]).[19]

More recently, Matsuo and his coworkers[20,21] have isolated two new natriuretic peptides. Brain natriuretic peptide (or B-type natriuretic peptide; BNP) and C-type natriuretic peptide (CNP) were both isolated from porcine brain extracts on the basis of their potent relaxant effects on chick rectum. Like ANP, these hormones are synthesized from large precursor proteins, and the mature, active peptides have a 17-amino-acid loop formed by an intramolecular disulfide linkage. In the human peptides (Figure 2), 11 of these amino acids are identical in ANP, BNP, and CNP, whereas the N- and C-terminal tails vary in both length and composition.[22-24] CNP has no C-terminal tail, and studies of the structure of the gene for CNP demonstrated that translation is terminated by a stop codon immediately after the final cysteine codon in the mRNA.[24-26]

Among species, the amino acid sequence of both ANP and CNP are highly conserved, whereas the structure of BNP varies greatly (Figure 3). For example, the mature 28-amino-acid human and porcine ANPs are identical, and there is only one substitution in the rat peptide.[10,11,27,28] Studies of the complementary DNA (cDNA) precursors for CNP indicate that the 22-amino-acid forms of porcine, rat, and human CNP are identical.[24-26] The N-terminally extended 53-amino-acid form of CNP is identical in porcine and rat CNP and has only two substitutions in human CNP. In nonmammalian tissue, a 22-amino-acid form of chicken CNP is identical to the mammalian form except for three amino acid substitutions.[29] The porcine, human, and rat BNPs, in contrast, are only 50% identical at the amino acid level, and the mature hormones vary in length from 26 to 45 residues.[30-32] This species variation is reflected in the biological activity of BNP studied both in vivo and in vitro. Although rat and human ANP are able to relax precontracted porcine coronary arterial strips with equal potency ($IC_{50} \approx 0.04$ nM), human BNP was much more potent than rat BNP in this assay ($IC_{50} = 0.02$ versus 1.10 nM, respectively).[33] These results suggest
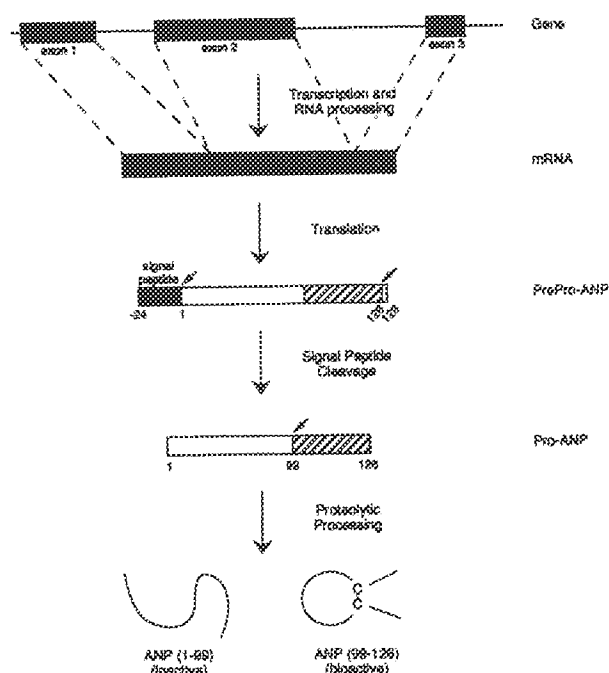
FIGURE 1. *Schematic of the synthesis of bioactive atrial natriuretic peptide (ANP) (99–126).*

that rat and human ANP, which differ in only one amino acid, are recognized equally well by the appropriate porcine receptor. Rat and human BNP have only 50% sequence identity, however, and this variation leads to a differential ability of these hormones to activate the porcine receptor.

Although the three natriuretic peptides have highly homologous structures, they have distinct sites of synthesis and presumably discrete functions. Recent studies have compared the tissue distribution of the immunoreactivelike material and mRNA coding for ANP, BNP, and CNP.[25,34,35] In porcine and human tissue, radioimmunoassays have demonstrated that both ANP and BNP are synthesized predominantly in the heart, with ANP concentrations approximately 45 times higher than BNP. Within the heart, the atrium is the primary site of synthesis for both hormones. The ventricle produces ANP and BNP, but at levels 1,000- and 100-fold less than the atrium, respectively. This difference in peptide concentrations is also reflected at the RNA level in the rat.[25] Northern blot analyses demonstrate a very high level of ANP mRNA in the atrium, and the difference in the levels of BNP mRNA in the atrium compared with the ventricle is much smaller than the difference in ANP mRNA concentrations in

those regions. During cardiac disease, the expression of both ANP and BNP increases dramatically in both the atrium and ventricle.[36] For BNP, the ventricle becomes the predominant site of hormone synthesis, and release into the anterior interventricular vein accounts for the increasing levels of circulating BNP. In patients with severe congestive heart failure, the plasma levels of both ANP and BNP are higher than control values; the increase in BNP concentrations, however, is at least 10 times greater than that of ANP.[36] These results suggest that ANP and BNP have distinct physiological and pathophysiological roles in cardiovascular control. Studies of infusions of BNP into patients with congestive heart failure support this hypothesis. Although the hemodynamic effects of BNP and ANP were similar in these patients, BNP had a longer duration of action and caused an enhanced rather than a blunted natriuretic response as was seen with ANP administration.[37]

Both ANP and BNP immunoreactivity has been found in the central nervous system and the adrenal medulla as well as in cardiac tissue.[34] In the porcine brain, BNP levels are approximately 10-fold higher than those of ANP. Because the concentration of both of these peptides in the nervous system is much lower than in the heart or in the circulation, it has been postulated that the biological actions of ANP and BNP are predominantly in the periphery. The presence of these peptides in the brain, however, has led to many studies of the possible roles of ANP and, to a lesser extent, BNP in the central control of various cardiovascular functions.[38] For example, injections of ANP or BNP into the rat brain cause changes in diuresis and salt appetite,[39–45] heart rate and blood pressure,[46,47] vasopressin secretion from the hypothalamus,[48,49] and angiotensin II–induced water intake.[41,50–52] These results suggest that one important physiological role for natriuretic peptides in the brain is the regulation of the activity of the vasopressin and angiotensin II hypothalamic systems to maintain proper body fluid homeostasis. Therefore, even though the concentrations of ANP and BNP in the brain are much lower than those in the heart, these natriuretic peptides probably do have roles as neurotransmitters.

CNP appears to be localized exclusively to the central nervous system or to cells derived from the neural crest. Radioimmunoassay results indicate that CNP levels are 1.5–10 times higher than ANP and BNP in porcine brain (0.79 versus 0.06 and 0.52 pmol/g, respectively)[34] and 30–70 times higher in human brain (1.04 versus 0.039 and 0.015 pmol/g, respectively).[35] The significant levels of CNP-like immunoreactivity detected in both porcine and human heart (1–5 pmol/g) have been shown to be caused completely by cross-reactivity with the extremely high concentration of ANP.[35] RNA blot
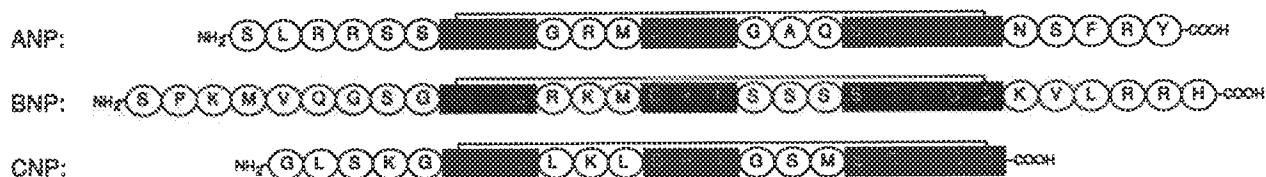

FIGURE 2. *Diagrammatic comparison of the mature human atrial (ANP), brain (BNP), and C-type (CNP) natriuretic peptides. Conserved residues of ANP,[22] BNP,[23] and CNP[24] are represented by darkened boxes. The line between the two cysteines in each peptide indicates a disulfide bridge.*
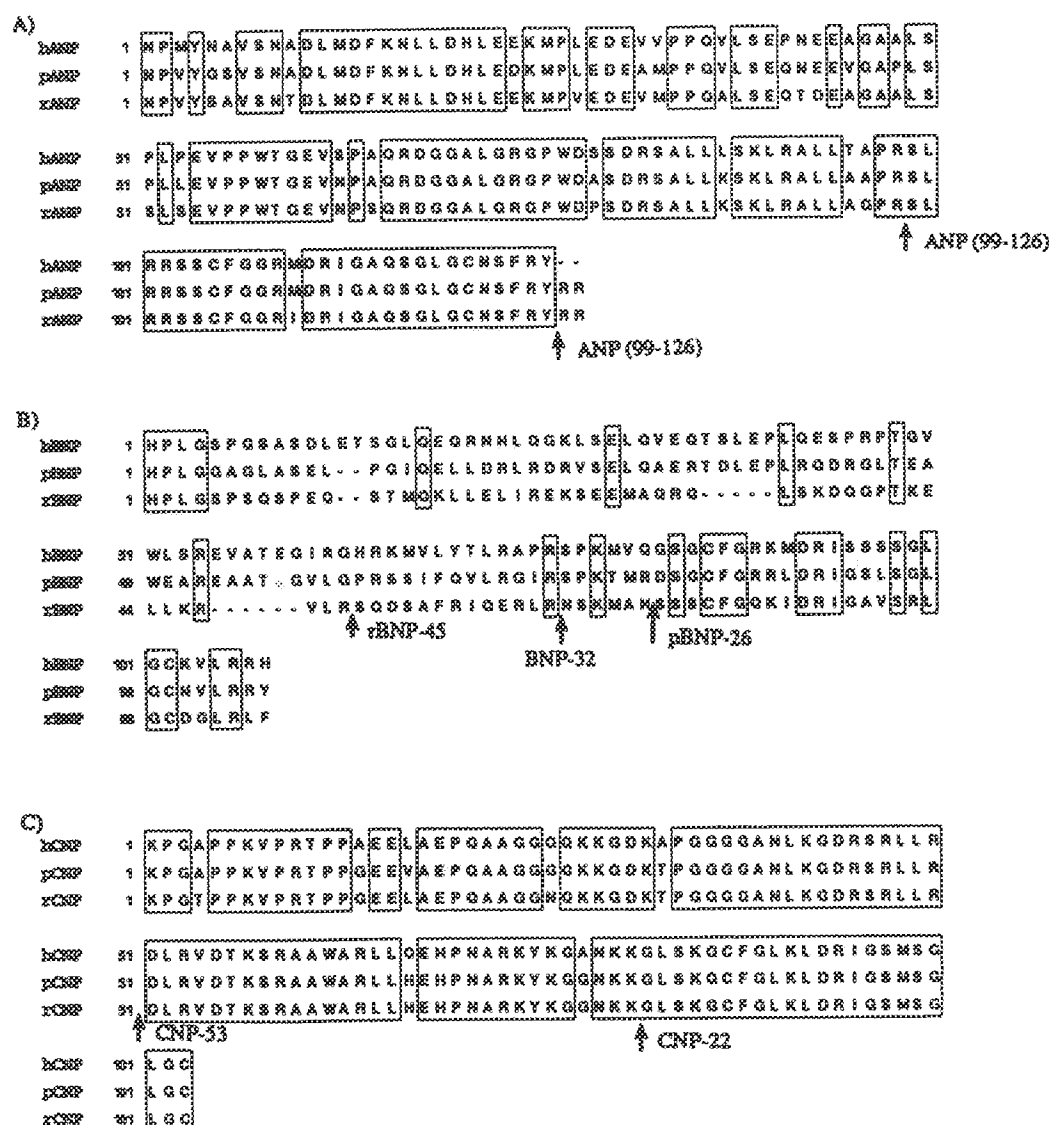
FIGURE 3.   *Diagrammatic comparison of the amino acid sequences of human, porcine, and rat atrial (ANP), brain (BNP), and C-type (CNP) natriuretic peptide prohormones. Panel A: Human,[27] porcine,[28] and rat[6-11] ANP prohormone sequences. Arrows indicate sites of processing for synthesis of mature ANP (99–126). Panel B: Human,[32] porcine,[30] and rat[31] BNP prohormone sequences. All three hormones can be cleaved to form BNP-32. The porcine and rat prohormones are also processed to yield pBNP-26 and rBNP-45, respectively. Panel C: Human,[24] porcine,[26] and rat[25] CNP prohormone sequences. Potential processing sites for both CNP-22 and CNP-53 are shown.*

analysis of various rat tissues confirms this result and identifies a major transcript for CNP solely in the brain.[25] No hybridization is seen to RNA from atrium, ventricle, lung, liver, stomach, or intestine. Although CNP has been shown to have natriuretic, diuretic, and hypotensive effects in vivo, it is significantly less potent than either ANP or BNP.[20,21] It is possible that CNP's primary biological activity is not as a classic natriuretic peptide with peripheral sites of action. The expression of the CNP gene exclusively in the nervous system[25] and its high neuronal concentration compared with ANP and BNP[35] suggest that CNP may act as a neurotransmitter to coordinate central aspects of salt and water balance and blood pressure. Further studies on the physiology of CNP may help clarify its role.

Natriuretic Peptide Receptors

Most of the biological activities of the natriuretic peptides are thought to be mediated by intracellular accumulation of guanosine 3',5'-monophosphate (cGMP) through the activation of particulate guanylyl cyclase.[53,54] Molecular cloning studies have identified three different natriuretic peptide receptors (NPRs; human receptors are shown schematically in Figure 4).[55-60] Two of these, NPR-A and NPR-B (also called GC-A and GC-B), are members of the newly described family of receptor guanylyl cyclases. These receptors are single transmembrane proteins whose extracellular domains are 44% identical. The intracellular regions of these proteins can be divided into two domains. The approximately 250-amino-acid C-terminal portion of
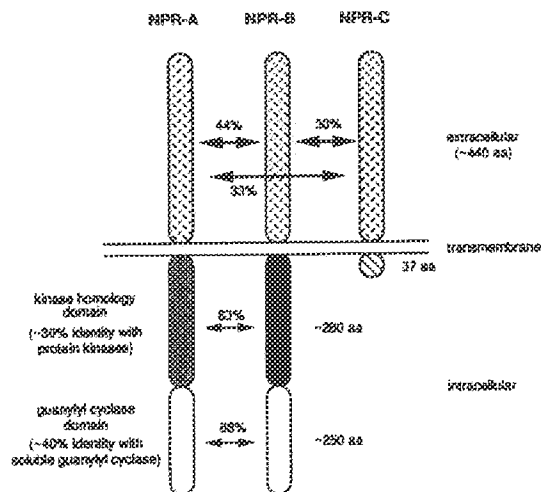
FIGURE 4. *Diagram of the natriuretic peptide receptor (NPR) family. Schematic of the three known receptors is presented. Percentages indicate percent sequence identity within protein domains for the human receptors. See text for details.*

NPR-A and NPR-B is the guanylyl cyclase catalytic domain, which is activated upon binding of the appropriate natriuretic peptide to the extracellular domain. This catalytic region has the highest amount of sequence identity between NPR-A and NPR-B (88%). The other known mammalian member of the receptor guanylyl cyclase family, the heat-stable enterotoxin receptor (STa R, GC-C or the guanylin[61] receptor) is 55% identical to NPR-A and NPR-B in this region.[62,63] In addition, this region is highly homologous to the soluble guanylyl cyclases[64,65] and to regions within adenylyl cyclase.[66]

The second intracellular domain in these proteins is the approximately 280 amino acids that immediately follow the transmembrane domain. This region (the kinase homology domain, or KHD) has only approximately 30% identity to both soluble and membrane-bound forms of protein kinases,[55-58,67] whereas NPR-A and NPR-B are approximately 68% identical in this region. However, more than 80% of the key invariant or conserved residues found in all protein kinases[67] are present in NPR-A and NPR-B. The functional significance of this homology to protein kinases is unclear. So far, no kinase activity has been detected in the guanylyl cyclase receptors.

The third member of the NPR family is NPR-C (Figure 4). Molecular cloning[59] showed that this receptor contains a very short 37-amino-acid cytoplasmic tail that bears no homology to the intracellular domain of any other known receptors. Its extracellular domain, however, is approximately 30% identical to NPR-A and NPR-B. Binding studies have demonstrated that NPR-C has a much less stringent specificity for structural variants of ANP than does NPR-A or NPR-B.[68] This pharmacological difference was among the first biochemical evidence to suggest heterogeneity within NPR subtypes. The physiological role for NPR-C is not clear. Maack et al[69] have postulated that this protein functions as a clearance receptor to remove large amounts of ANP from the circulation or to store ANP
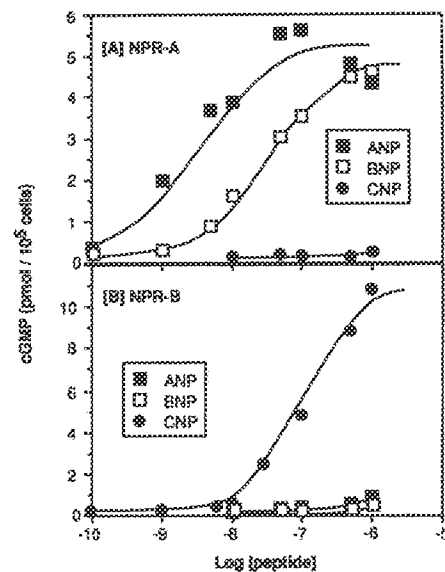

FIGURE 5. *Graphs showing concentration-dependent stimulation of natriuretic peptide receptors NPR-A and NPR-B by natriuretic peptides. Tissue culture cells transiently expressing human NPR-A (panel A) or NPR-B (panel B) were treated with increasing concentrations of human natriuretic peptides, and the levels of intracellular cGMP were determined. ANP, BNP, CNP, atrial, brain, and C-type natriuretic peptides.*

and release it slowly. In fact, this receptor is quite abundant in vascular tissue, where it may serve to absorb excess ANP and protect against the sudden onset of dangerously low blood pressure. Recent evidence, however, has suggested that NPR-C may mediate the biological effects of ANP through second messengers other than cGMP. For example, analogues of ANP known to interact specifically with NPR-C have been shown to inhibit the production of cyclic AMP (cAMP) in various tissues[70] and activate the phosphoinositol pathway in aortic smooth muscle cells.[71] However, experiments measuring various signal transduction pathways in cells expressing the cloned human NPR-C have not yet been successful.[72]

The three NPR subtypes each recognize the three known natriuretic peptides differentially. Dose-response curves for stimulation of the guanylyl cyclase of NPR-A and NPR-B demonstrate that these receptors can be selectively activated (Figure 5).[73,74] Tissue culture cells transiently expressing the cloned human receptors were stimulated with various concentrations of the human hormones, and intracellular cGMP levels were determined. Both ANP and BNP can effectively stimulate NPR-A; BNP, however, is approximately 10-fold less potent. CNP, in contrast, does not significantly increase intracellular cGMP in cells expressing NPR-A. For NPR-B–expressing cells, only CNP can effectively stimulate the receptor's enzymatic activity. Neither ANP or BNP increased cGMP levels in these cells except at very high, nonphysiological concentrations.

Binding studies by Bennett et al[75] on the extracellular domains of the receptors show a correlation between the ability of the natriuretic peptides to stimulate guanylyl cyclase activity and the affinities of these hormones for the binding domains of the receptors

TABLE 1. Affinity of the Human Natriuretic Peptide Receptors for the Three Human Natriuretic Peptides

Receptors	Dissociation constants		
	ANP	BNP	CNP
NPR-A*	1.9 pM	7.3 pM; 0.14 nM‡	>500 nM
NPR-B†	5.4 nM	30 nM	7 pM
NPR-C*	2.6 pM	13 pM	10.8 pM

All values determined from experiments using the extracellular domain of each receptor fused to the constant domain of IgG (see Reference 75). ANP, BNP, CNP, atrial, brain, and C-type natriuretic peptides.

*Experimental data from displacement experiments using ^{125}I-hANP.

†Experimental data from displacement experiments using ^{125}I-hCNP.

‡The data obtained for hBNP binding to NPR-A were best represented as a two-site model.

(Table 1). Both ANP and BNP can bind NPR-A with relatively high affinity, but ANP is 4–70 times more potent than BNP. This result corresponds well with the 10-fold difference in EC_{50}s of these hormones to stimulate NPR-A cyclase activity. In addition, CNP also does not effectively bind to NPR-A, even at very high concentrations. For NPR-B, only CNP can bind with extremely high affinity. NPR-B's affinity for CNP is approximately three orders of magnitude greater than for either ANP or BNP. Therefore, the rank order of potency of the natriuretic peptides for binding to and for stimulation of NPR-A and NPR-B is identical. All three of the human hormones and a remarkable diversity of natriuretic hormones and analogues[68] bind NPR-C with high affinity. This result could be consistent with a possible role for NPR-C as a hormonal buffering system[69] to regulate circulating levels of all forms of natriuretic peptides.

Because the NPR-A guanylyl cyclase is activated by both ANP and BNP, and both hormones have similar physiological effects in vitro[20,54] and in vivo,[78] it is possible that these two cardiac hormones exert their actions primarily through the same receptor, i.e., NPR-A. However, recent in vivo infusion experiments[57] and studies of ANP and BNP levels[56] in patients with congestive heart failure indicate that these two hormones have discrete physiological roles (see discussion above) and, therefore, may interact with distinct receptors. Perhaps there is an as yet unidentified member of the NPR guanylyl cyclase family that is specifically activated by BNP. Because the sequences of ANP and CNP are highly conserved across species, as are the sequences of their receptors, NPR-A and NPR-B,[55–58] respectively, it is possible that a BNP-specific receptor exists that displays as much structural diversity among species as does BNP itself.

Studies on the localization of the NPRs have been hampered by the lack of good antibodies for immunohistochemical studies. Receptor autoradiographic experiments using ^{125}I-ANP to evaluate binding in various tissues[77] have the drawback that this technique does not distinguish between the subtypes of NPRs, because both NPR-A and NPR-C bind ANP with high affinity. Recently, the differential expression of the mRNA for the three receptors has been examined in primate tissue
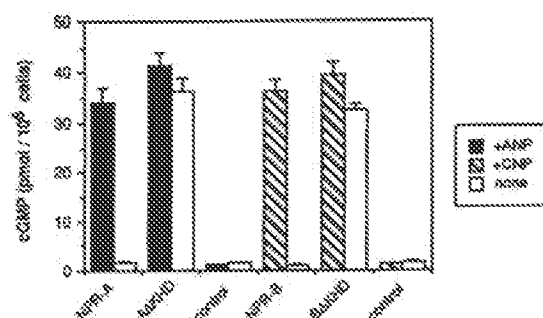

FIGURE 6. *Bar graphs showing effect of the deletion of the kinase homology domain (KHD) on the activity of the human natriuretic peptide receptor (NPR) guanylyl cyclases. COS cells expressing the wild type (NPR-A and NPR-B) or kinase homology domain deleted (AΔKHD and BΔKHD) receptors or control cells were stimulated with 0.5-μM concentrations of the indicated human natriuretic peptide, and intracellular cGMP was measured. ANP, CNP, atrial and C-type natriuretic peptides.*

by use of in situ hybridization.[72] These studies suggest a correlation between the known sites of actions of the hormones and the sites of receptor synthesis. For example, NPR-A mRNA is present in the kidney, adrenal, heart, and to a lesser extent, brain. NPR-B mRNA, on the other hand, appears to be localized solely to neuronally derived tissue such as the brain, pituitary, and adrenal medulla, as is its ligand, CNP. No specific hybridization was seen in the heart or kidney. The mRNA encoding NPR-C is found in the heart, kidney, and brain as well as within discrete clusters of cells throughout the adrenal cortex and medulla.

Molecular cloning experiments demonstrated conclusively that two of the receptors for the natriuretic peptides (NPR-A and NPR-B) contained the protein moiety necessary for synthesizing their second messenger, cGMP, on the same polypeptide as the binding domain.[55–58] This result has renewed the interest of many investigators in understanding the molecular basis for the mechanisms of regulating the guanylyl cyclase activity of these receptors. Studies of NPR-A, and more recently NPR-B, have suggested that the KHD has an important role in transducing the signal generated by hormone binding; e.g., stimulation of cGMP production.[78,79] ATP has been shown to be essential for or to cause potentiation of the cyclase activity of NPR-A.[80,81] It is attractive to speculate that ATP acts by interacting with the KHD, because this region contains a glycine-rich nucleotide binding motif. Mutant NPR proteins in which the KHD has been deleted (AΔKHD or BΔKHD) constitutively produce cGMP,[78,79] independent of the binding of natriuretic peptides (Figure 6), and ATP no longer modulates the enzymatic activity of AΔKHD.[78] These results suggest that the KHD acts as a negative regulator of cyclase activity and that hormone binding to the extracellular domain releases this inhibition. The tyrosine kinase activity of the epidermal growth factor receptor (EGF R) appears to be regulated in a similar manner. The C-terminus of this receptor interacts with the catalytic domain to suppress phosphorylation.[82] EGF binding causes a conformation
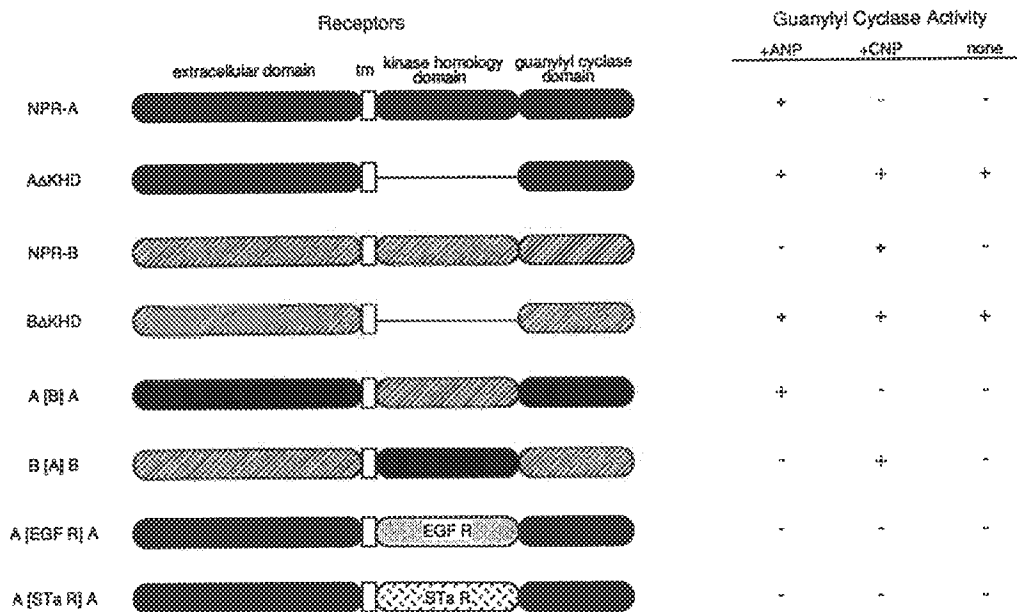
FIGURE 7. *Diagram of guanylyl cyclase activity of chimeric receptors. Chimeric receptors generated by altering the cDNA for these proteins were expressed in tissue culture cells and stimulated with human natriuretic peptides. EGF R, epidermal growth factor receptor; STa R, heat-stable enterotoxin receptor. The ability of these receptors to synthesize cGMP after hormone binding is indicated by + or −. See text for details. NPR, natriuretic peptide receptor; ANP, CNP, atrial and C-type natriuretic peptides; tm, transmembrane domain.*

change, receptor dimerization, and stimulation of the receptor's enzymatic activity.[83]

Chimeric receptors in which the KHDs have been exchanged have been used to study the role of this domain in the regulation of the guanylyl cyclase activity of NPR-A and NPR-B.[79] When the kinaselike domains were exchanged between NPR subtypes, no loss in regulation of guanylyl cyclase activity was detected (A[B]A and B[A]B in Figure 7). The KHD of NPR-A could correctly regulate the cyclase activity of NPR-B and vice versa. In addition, the ligand specificity of the receptor was not changed by the presence of the alternative domain; i.e., both NPR-A and A[B]A were stimulated by ANP and not CNP, and NPR-B and B[A]B were activated only by CNP.

Chimeric proteins made with homologous portions of structurally related receptors, however, were inactive (Figure 7). When the KHD of NPR-A was replaced with the kinase domain of the EGF R (A[EGF R]A) or the KHD of the STa R (A[STa R]A), the resultant proteins were unable to correctly regulate the guanylyl cyclase activity. One possible explanation for these results is that although the identity between the kinaselike domains of NPR-A and NPR-B is 60%, these two NPRs are only 30% identical to either the STa R or the EGF R in the same region. These types of experiments indicate that the KHD is obviously an important regulator of the enzymatic activity of receptor guanylyl cyclases and may be the site of action for the effect of ATP, but understanding of the mechanism of this regulation awaits further study. Perhaps site-specific mutations within the kinaselike domain will help answer this question.

Summary

After the description in the past 5 years of BNP and CNP, interest in the natriuretic peptide family has dramatically increased. Molecular characterization of the receptors for this hormone family has identified a heterogeneity in the receptor subtypes not previously alluded to by pharmacological or biochemical studies. Much has been published on the physiology of ANP, but the major roles for BNP and CNP remain to be elucidated. Some experiments indicate that ANP and BNP may act synergistically, especially during cardiac stress[36,37]; however, the high level of structural diversity of BNP among species and the ability of porcine BNP, but not human BNP, to activate human NPR-B[73] suggest that an as yet unidentified receptor may exist that specifically recognizes BNP. Localization studies have implied that CNP is the most prominent neuropeptide in the natriuretic peptide family, and the restriction of its receptor, NPR-B, to the nervous system suggests that CNP and NPR-B may act in the brain to coordinate the central aspects of body fluid homeostasis.

Of the three known NPRs, two, NPR-A and NPR-B, are capable of synthesizing their own second messenger, cGMP. The domain within these receptors that has high homology to protein kinases has been demonstrated to be essential for regulating this activity. No kinase activity has been measured in these proteins, but it is possible that this region is important for ATP regulation of guanylyl cyclase activity. This possibility raises interesting parallels with receptor-mediated cAMP signaling within cells.[84] Seven transmembrane receptors, once activated by ligand, associate with G proteins to affect the activity of adenylyl cyclase. This process is modulated by guanine nucleotide binding to the intermediate G protein. Perhaps guanylyl cyclase activity is regulated in a similar manner by adenine nucleotide binding to the kinaselike domain of the NPRs. The third type of natriuretic peptide receptor, NPR-C, is the most abundant and most widely distributed in various tissues. It

has no known enzymatic activity, but it can bind all three natriuretic peptides, as well as a wide range of structurally related analogues, with high affinity. The physiological role for this receptor is still unclear; it may serve as a clearance receptor to protect against acute hypotension, or it may signal through second messenger pathways other than cGMP.

The characterization of the natriuretic peptide and receptor families suggests a complex mechanism for the body's control of fluid homeostasis and blood pressure. A more complete understanding of the physiological roles of the members of these families may help in the development of new therapeutic agents to control such pathophysiological states as hypertension, congestive heart disease, and renal failure.

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Exhibit B
Sallusto and Lanzavecchia (2002)
"The instructive role of dendritic cells on T-cell responses."
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Supplement Review
The instructive role of dendritic cells on T-cell responses

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Chapter summary

Immune responses are initiated in the T-cell areas of secondary lymphoid organs where naïve T lymphocytes encounter dendritic cells (DCs) that present antigens taken up in peripheral tissues. DCs represent the interface between the universe of foreign and tissue-specific antigens and T lymphocytes, and they are the key players in the regulation of cell-mediated immunity. We discuss how the nature of the DC maturation stimuli and the density and quality of DCs present in the T-cell areas of secondary lymphoid organs determine the magnitude and class of the T-cell response.

Keywords: dendritic cells, effector and memory T cells, T-cell activation, T-cell tolerance

Introduction

DCs possess specialized features, such as pathogen recognition, antigen capturing and processing machinery, migratory capacity and costimulatory molecules, that allow them to act as the professional antigen presenting cells (APC) [1]. DCs represent the interface between the universe of foreign and tissue-specific antigens and T lymphocytes. They also play a role in all aspects of T-cell responses, from the deletion of self-reactive thymocytes to the generation of effector and memory cells, as well as the induction of peripheral tolerance.

In this review, we shall discuss how DCs provide a qualitative and quantitative framework for T-cell antigen recognition. We shall first summarize the requirements for T-cell activation and differentiation in terms of concentration of peptide–MHC complexes, costimulatory molecules and cytokines. We shall then consider how DCs assemble these components and deliver them, as discrete short-lived packets, to the T-cell areas. Finally, we shall discuss how the nature of the DC maturation stimuli determines the density and quality of antigen-carrying DCs and, consequently, the magnitude and class of T-cell responses.

Activation and differentiation of naïve T lymphocytes

The signals that lead to T-cell activation are generated at the level of the immunological synapse, a specialized area of contact between T cells and APC where adhesion molecules and TCRs segregate into distinct supramolecular complexes [2,3]. At the synapse, the TCRs are sequentially triggered by peptide–MHC complexes, a process that allows the signal to be sustained for as long as the synapse is in place [4,5]. Synapses are stable in the absence of disturbing influences, but they can be disrupted by cell division, by death of APC or by external influences, such as collagen or chemokines. T cells continuously search for antigen and can rapidly shift from one APC to another offering a higher level of stimulation. While the duration of TCR stimulation depends on the duration of the synapse, the intensity of signal that T cells receive is dependent both on the level of peptide–MHC complexes

A glossary of specialist terms used in this chapter appears at the end of the text section.

that trigger TCRs and the level of costimulatory molecules that amplify the signalling process [6].

The efficiency of signal transduction varies with the nature of the APC and the T cell's developmental stage [6,7]. In activated, effector and memory T cells, TCR triggering is efficiently coupled to signal transduction pathways so that the cells can respond to low doses of antigens even in the absence of costimulation. In contrast, TCRs are inefficiently coupled in naïve T cells. Engagement of CD28 by B7 molecules expressed by professional APC recruits membrane rafts containing kinases and adapters to the synapse, and amplifies up to 100-fold the signalling process initiated by the TCRs. In the absence of costimulation, naïve T cells can thus be activated only by extremely high (nonphysiologic) doses of antigens and they require a prolonged stimulation, while in the presence of costimulation they can respond to ~100-fold lower doses of antigen and can also respond more rapidly. Depending on the antigen dose and the level of costimulation, naïve T cells require between 6 and >30 hours of TCR stimulation to become committed to cell division, while memory/effector T cells respond within 0.5–2 hours [7].

Once committed to division, T cells proliferate rapidly in response to IL-2, which is produced in an autocrine or paracrine fashion by activated T cells. We have shown that the duration of TCR stimulation together with polarizing cytokines determines the progressive differentiation of CD4+ and CD8+ T cells, leading to the generation of terminally differentiated effector cells as well as intermediates [8,9]. T cells that receive a short TCR stimulation proliferate, but fail to differentiate to effector cells and retain the lymph-node homing capacity characteristic of naïve T cells. In contrast, T cells that receive a prolonged TCR stimulation in the presence of IL-12 or IL-4 differentiate to Th1 or Th2 cells. As part of their differentiation programme, Th1 and Th2 cells lose the lymph-node homing receptors and acquire receptors that control their migration to inflamed nonlymphoid tissues where they can execute effector functions.

T cells interact with DCs in a highly dynamic environment where they have to compete to achieve a level of TCR stimulation sufficient to drive their activation and differentiation processes. We suggest that the progressive process of T-cell differentiation combined with the stochastic stimulation of proliferating T cells by random T cell–DC interactions leads to the generation of both intermediates and terminally differentiated cells within the same responding clone (Fig. 1) (F Sallusto, unpublished data). We consider this intraclonal diversification as a fundamental property of the immune system since, on the one hand, it prevents clonal exhaustion and, on the other, it allows the generation of distinct T-cell subsets that play a role in effector and memory responses [5]. The intraclonal
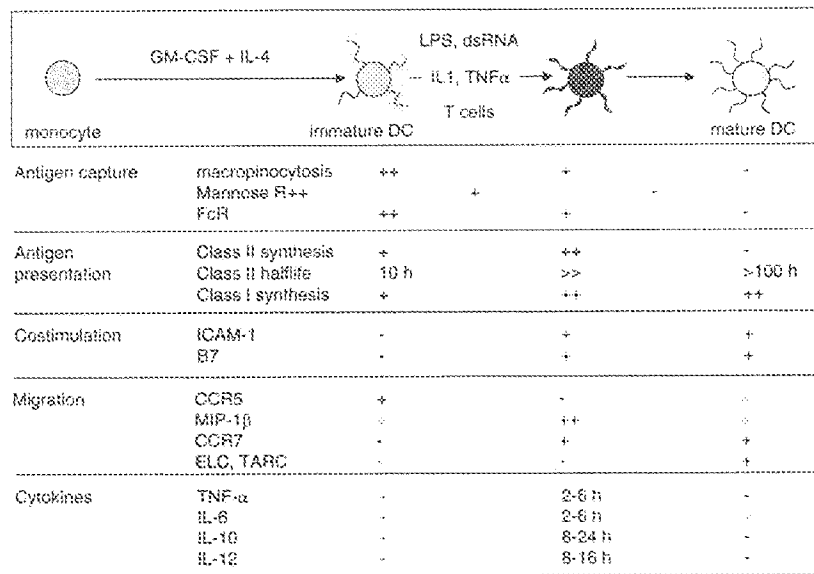
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Figure 1
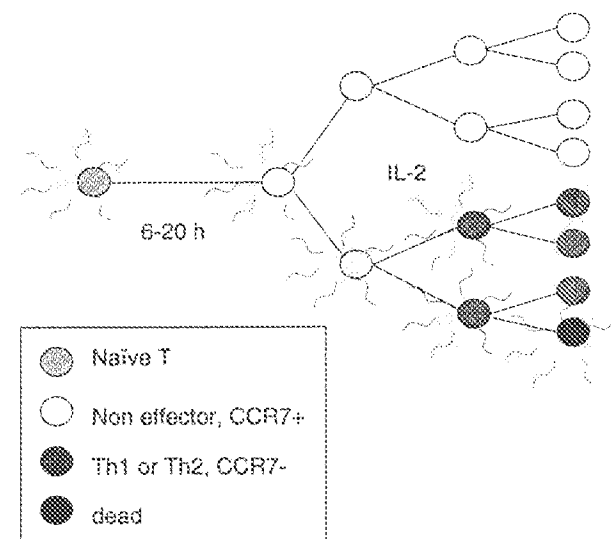
Stochastic stimulation of proliferating T cells leads to intraclonal functional diversification. By establishing immunological synapses with dendritic cells (DCs), naïve T cells (green) achieve stimulation and become committed to proliferate in response to autocrine or paracrine IL-2. T-cell receptor stimulation is sustained by serial encounters with DCs and, in the presence of polarizing cytokines (IL-12 and IL-4, not shown), drives T-cell differentiation to Th1 or Th2 effector cells that have lost CCR7 expression (red). T cells receiving a shorter stimulation do not acquire effector function and do retain lymph-node homing capacity (yellow). An excessive stimulation leads to activation-induced cell death (black).

differentiation model is supported by the existence of distinct subsets of memory cells: 'central memory' cells that represent intermediates, and 'effector memory' cells that represent terminally differentiated cells [10].

The DC maturation process

DCs that migrate from tissues to lymph nodes have a life expectancy of only a few days and can therefore be viewed as disposable packets, each carrying a given amount of peptide–MHC complexes, costimulatory molecules and cytokines. These packets are assembled during DC maturation, a process that is initiated by pathogens and/or inflammatory stimuli. The production of homogeneous populations of human immature DCs from human peripheral blood monocytes cultured with granulocyte/macrophage-colony-stimulating factor and IL-4 [11] has been instrumental in identifying the maturation stimuli and in dissecting the DC maturation process (Fig. 2).

DC maturation is triggered and modulated by a variety of receptors for microbial products, cytokines and T cells [12,13]. Human monocyte derived and myeloid DCs express several Toll-like receptors such as TLR2 and TLR4 that trigger maturation in response to bacterial peptidoglycan

Figure 2

		immature DC			mature DC
Antigen capture	macropinocytosis	++		+	-
	Mannose R++		+	-	
	FcR	++		+	-
Antigen presentation	Class II synthesis	+		++	-
	Class II halflife	10 h		>>	>100 h
	Class I synthesis	+		++	++
Costimulation	ICAM-1	-		+	+
	B7	-		+	+
Migration	CCR5	+		-	-
	MIP-1β	-		++	-
	CCR7	-		+	+
	ELC, TARC	-		-	+
Cytokines	TNF-α	-		2-6 h	-
	IL-6	-		2-6 h	-
	IL-10	-		8-24 h	-
	IL-12	-		8-16 h	-

The maturation programme studied in monocyte-derived dendritic cells. DC, dendritic cells; ELC, endothelial-like cells; FcR, receptors for crystallizable fragment [of antibody]; GM-CSF, granulocyte/macrophage-colony-stimulating factor; ICAM-1, intracellular adhesion molecule-1; LPS, lipopolysaccharide; MIP, macrophage inflammatory protein; TARC, thymus- and activation-regulated chemokine.

and lipopolysaccharide, respectively. Interestingly, these receptors are absent in plasmacytoid DCs (also known as interferon-producing cells [IPC]) that instead express TLR9, which mediates the response to CpG DNA [14,15]. The differential responsiveness of myeloid and plasmacytoid DCs to pathogens underlines a division of labour between human DC subsets. DC maturation can be also triggered by tumour necrosis factor (TNF)-α and IL-1, and can be inhibited by IL-10. Finally, all DC types are exquisitely sensitive to T-cell feedback signals delivered by activated T cells through CD40 ligand (CD40L).

The maturation process coordinately regulates antigen capturing, processing and presentation, expression of costimulatory molecules, cytokine production and lifespan. Immature DCs are extremely efficient in antigen capture since they possess high levels of constitutive macropinocytosis and express endocytic receptors for microbial patterns, such as the mannose receptor [16]. Maturation increases synthesis of MHC class II molecules, while decreasing their degradation, thus favouring the rapid accumulation of long-lived peptide–MHC complexes, which are retained for several days, while class II synthesis is shut off [17]. Presentation on MHC class I molecules is also enhanced by an approximately 10-fold increase in the rate of synthesis, which is sustained in mature DCs [18]. DCs are capable of transporting phagocytosed antigens from the endocytic compartment to the cytosol, leading to their 'cross-presentation' to CD8+

T cells [19,20]. Finally, maturation stimuli upregulate the expression of B7.1 and B7.2, thus enhancing the T-cell stimulatory capacity of DCs. While the upregulation of MHC molecules ensures higher capacity for antigen presentation, upregulation of costimulatory molecules ensures an efficient amplification of signalling in naïve T cells.

Cytokine production by DCs is subject to a tight regulation, which is particularly relevant in the case of IL-12 and IFN-I, the major Th1-polarizing cytokines [21]. IL-12 production is elicited by most pathogens and is boosted by activated T cells through CD40L [22]. In contrast, IL-12 is not induced by other maturation stimuli such as TNF-α, IL-1, and cholera toxin. IL-12 production can be modulated by cytokines and mediators present during induction of maturation: IFN-γ and IL-4 enhance IL-12 production induced by appropriate stimuli, while prostaglandin E_2 and IL-10 exert an inhibitory effect. In addition, one has to consider that IL-12 production is restricted to a narrow temporal window, 8–16 hours after induction of DC maturation [9]. In summary, the Th1-polarizing capacity of DCs is contingent on a number of variables that include the lineage of DCs, the microenvironment in which they are stimulated, the maturation stimuli, and the kinetics of maturation (Fig. 3).

Dynamic changes in DC type and concentration impact on T-cell responses

Because the half-life of mature DCs is short and because cytokine production is transient, the number and type of

Figure 3
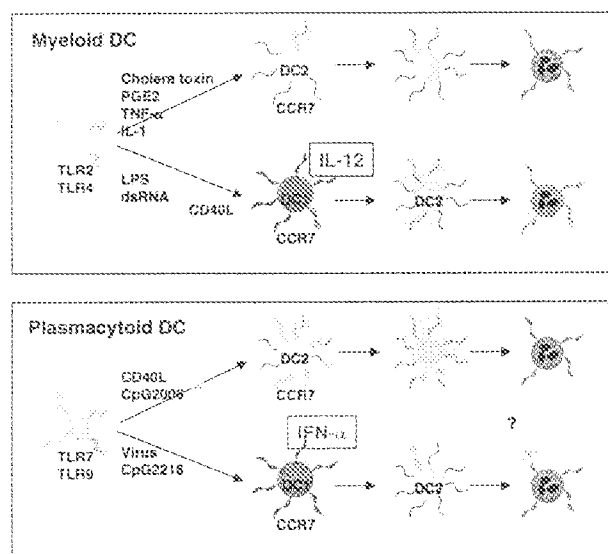
Reactivity, flexibility, kinetics and exhaustion in myeloid and plasmacytoid dendritic cells (DCs). The figure summarizes the properties of immature myeloid and plasmacytoid DCs. Indicated are the most relevant Toll-like receptors (TLR) and chemokine receptors, and the response to various maturation stimuli. DC1 and DC2 refer to the capacity of the cells to induce Th1 and Th2 responses, respectively. CD40L, CD40 ligand; LPS, lipopolysaccharide; PGE2, prostaglandin E_2

DCs present in the T-cell areas will reflect, in highly dynamic fashion, the conditions of the tissues from which the lymph is drained. Under steady-state conditions, only a few tissue-resident DCs 'spontaneously' mature and migrate to the draining lymph nodes, carrying antigens and apoptotic bodies taken up in peripheral tissues [23]. These migrating DCs do not induce effector responses, but rather trigger an abortive T-cell proliferation that leads to immunological tolerance [24,25]. It is possible that spontaneously matured DCs deliver to T cells a qualitatively distinct tolerizing signal. Alternatively, according to the progressive differentiation model, we suggest that the low frequency and short lifespan of these DCs, together with the low level of antigen and B7, may deliver a weak stimulus to T cells, which is not sufficient to sustain proliferation and to promote differentiation.

When pathogens (or adjuvants) are present in peripheral tissues, resident DCs are activated *en masse* and migrate to the draining lymph nodes. At the same time monocytes are recruited from peripheral blood into inflamed tissues, where they rapidly differentiate to DCs that capture antigens and, on maturation, migrate to the draining lymph nodes. This mechanism sustains antigen sampling and presentation for extended periods of time. Maturing DCs

produce large amounts of inflammatory cytokines and chemokines that promote monocyte recruitment [26]. The relative role of tissue-resident DCs, such as Langerhans cells and dermal DCs, versus recruited DCs, such as monocyte-derived DCs and IPC, remains to be established. Production of IFN-I by IPC may be important to promote maturation of monocytes and to protect them from the cytopathic effects of viruses [27,28].

In summary, under inflammatory conditions, the T-cell areas of draining lymph nodes receive large numbers of highly stimulatory DCs for a sustained period of time. The high DC density and the high levels of antigen and B7 molecules deliver a strong and sustained stimulation to specific T cells, leading to their rapid proliferation and differentiation. High levels of IL-2 are produced under these conditions and drive clonal expansion of committed T cells irrespective of whether they continue to receive TCR stimulation. One should also consider that DC–T cell interaction results in a reciprocal stimulation. Activated T cells trigger DCs via CD40L or TNF-related activation-induced cytokine, improving their T-cell stimulatory capacity, boosting IL-12 production, and prolonging their lifespan [29]. It is possible that regulatory T cells may suppress antigen presentation by DCs via production of inhibitory cytokines or by direct contact [30].

There is growing evidence that the capacity of DCs to induce Th1 or Th2 responses is contingent on appropriate stimulation and timing (Fig. 3). As already discussed, myeloid DCs produce IL-12 only in response to some pathogens or CD40L, and within a narrow time window. In addition, IPC produce large amounts of IFN-I, another Th1-polarizing cytokine, in response to viruses but not in response to CD40L; again, only within a narrow time window. In contrast, Th2 responses may be induced by DCs that do not produce Th1-polarizing cytokines, either because they have been conditioned by nonpermissive stimuli or because they have exhausted their IL-12 or IFN-I-producing capacity. In this case, Th2 polarization is driven by IL-4 produced by T cells themselves or derived from exogenous sources, such as natural killer T cells or mast cells. It is worth considering that the dynamics of DC migration to the draining lymph nodes may lead to preferential generation of Th1 cells during the early phases of the immune response, when active DCs enter the T-cell areas in large numbers. This is followed by induction of Th2 and nonpolarized T cells at later time points when the influx of DCs ceases and the DCs surviving in the T-cell area exhaust their IL-12-producing capacity [31].

Competition for DC shaping T-cell responses

The availability of antigen-presenting DCs and of antigen-specific T-cell precursors represents the limiting factors in the immune responses. There is growing evidence that responding T cells compete *in vivo* for access to DCs and

that this competition can be relieved by providing more DCs [32]. At the initial phase of a primary response, the low frequency of naïve T cells specific for a given antigen makes competition among responding cells unlikely. However, as the responding cells proliferate, competition for sustained TCR stimulation will increase, particularly among cells of the same clone, which have the same avidity and occupy the same niche. This intraclonal competition contributes to functional diversification: T cells achieving a sustained stimulation differentiate to effector cells, while those receiving a short stimulation remain in an intermediate state giving rise to central memory T cells. In contrast, interclonal competition may take place preferentially in secondary responses due to the larger numbers of antigen-specific cells present, and may therefore explain the selection of high-avidity T cells under these circumstances.

Conclusions

It is becoming increasingly clear that DCs provide the adaptive immune system with the essential function of context discrimination. DCs can integrate multiple stimuli from pathogens, inflammatory cytokines and T cells, and can provide distinct outputs in terms of antigen presentation, costimulation and cytokine production. Like other cells involved in the innate immune response, DCs produce large amounts of inflammatory chemokines that contribute to the recruitment of DC precursors in inflamed tissues, thus sustaining antigen sampling in peripheral tissue and presentation to T cells in lymph nodes. Finally, the T-cell activation and differentiation programme translates antigen concentration, cytokine and costimulatory molecule composition, and DC density into distinct cell fates ranging from tolerance to inflammation, cytotoxicity and memory.

Glossary of terms

APC = antigen presenting cells; CD40L = CD40 ligand; DC = dendritic cell; IPC = interferon-producing cells.

Acknowledgement

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EXHIBIT C
Giordano, *et al.* (2006)
"Nitric oxide and cGMP protein kinase (cGK) regulate dendritic-cell migration toward the
lymph-node–directing chemokine CCL19."
<u>Blood</u> **107**(4): 1537–1545

Nitric oxide and cGMP protein kinase (cGK) regulate dendritic-cell migration toward the lymph-node–directing chemokine CCL19

Daniela Giordano, Dario M. Magaletti, and Edward A. Clark

Dendritic-cell (DC) migration to secondary lymphoid organs is crucial for the initiation of adaptive immune responses. Although LPS up-regulates CCR7 on DCs, a second signal is required to enable them to migrate toward the chemokine CCL19 (MIP-3β). We found that the nitric oxide (NO) donor NOR4 provides a signal allowing LPS-stimulated DCs to migrate toward CCL19. NO affects DC migration through both the initial activation of the cGMP/cGMP kinase (cGMP/cGK) pathway and a long-term effect that reduced cGK activity via negative feedback. Indeed, migration of DCs toward CCL19, unlike migration toward CXCL12 (SDF-1α), required inhibition of cGK. LPS increased both cGK expression and cGK activity as measured by phosphorylation of the key cGK target vasodilator-stimulated phosphoprotein (VASP). Because cGK phosphorylation of VASP can disrupt focal adhesions and inhibit cell migration, LPS-induced VASP phosphorylation may prevent DCs from migrating without a second signal. Long-term NOR4 treatment inhibited the increase in cGK-dependent VASP phosphorylation, releasing this brake so that DCs can migrate. NO has been implicated in the regulation of autoimmunity through its effect on T cells. Our results suggest that NO regulation of DC migration and cytokine production may contribute to the protective effects of NO in autoimmune disorders. (Blood. 2006;107:1537-1545)

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Introduction

Dendritic cells (DCs) activate naive T cells in peripheral lymphoid tissues and play a pivotal role in initiating and instructing adaptive immune responses.[1,2] At the site of infection where they take up antigens, DCs also contribute to innate immunity: They recognize and respond to common pathogen-associated molecular patterns (PAMPs) by releasing proinflammatory products that regulate other inflammatory cells.[3] In a later phase, recognition of PAMPs triggers a complex maturation program that results in the increase in DC antigen-presenting ability and responsiveness to chemokines like CCL19/MIP-3β, which promote DC migration from peripheral tissues to secondary lymphoid organs.[4,5]

The outcome of an immune response depends not only on the pathogen that activates DCs but also on various inflammation-associated factors that modulate DC maturation and determine whether DCs polarize T-cell development into T helper 1 (Th1)–, Th2-, or regulatory T-cell subsets.[6] Prostaglandin E2 (PGE2) and other inflammatory products that act through the cAMP/cAMP kinase (cAMP/cAK) signaling pathway can affect DC maturation and drive them toward a Th2-inducing program.[7-9] These agents also regulate DC chemokine and chemokines receptor expression; they down-regulate receptors for inflammatory chemokines like CCR5 while increasing CCR7 or CXCR4 receptors for CCL19/MIP-3β and CXCL12/SDF-1α, respectively.[10-12]

Interestingly, inflammatory agents are required for mature DCs to be able to migrate toward certain chemokines. Even after monocyte-derived immature DCs (iDCs) are induced to mature and up-regulate CCR7, they remain unresponsive to CCL19 and do not migrate unless they are exposed to an inflammatory stimulus like PGE2 or ATP.[12-15] The importance of inflammatory factors for DC migration to lymph nodes (LNs) has been underscored recently.[16,17] EP4-deficient mice have Langerhans cells defective in their ability to migrate to LNs and impaired contact hypersensitivity immune responses.

Nitric oxide (NO) is a free radical gas produced during the development of inflammation by many cells, including macrophages, endothelial cells, and granulocytes.[18] Although NO can be toxic and proinflammatory when produced in large amounts, it may also regulate adaptive immune responses.[18,19] NO protects human and mouse DCs against apoptosis in models of sepsis.[20] NO also inhibits IL-12 and regulates MHC distribution during the maturation of mouse DCs,[21,22] but the role of NO in human DC maturation is still unclear.

Because NO acts principally through the cGMP/cGMP kinase (cGMP/cGK) signaling pathway, which often has effects similar to cAMP/cAK,[23] we hypothesized that NO might also be an important regulator of DC migration. We found that the addition of the NO donor, NOR4, during DC maturation enables DCs to migrate toward CCL19. We investigated the mechanisms by which NO couples CCR7 to the signal transduction involved in migration and, in particular, the cytoskeletal protein vasodilator-stimulated phosphoprotein (VASP), a target of cGK involved in cytoskeletal rearrangements occurring during cell migration.[24-26] Here we show that LPS stimulation induced a large increase in cGK expression and activity in DCs, leading to increased cGK-dependent VASP

The online version of this article contains a data supplement.

phosphorylation that impairs the ability of DCs to migrate. The presence of NO during DC maturation as a long-term effect decreased cGK-mediated phosphorylation of VASP. Thus, the enhanced ability to migrate induced by NO may be due in part to an increase in the amount of unphosphorylated VASP available to help form focal adhesions once DCs are stimulated by chemokines.

Materials and methods

Generation of iDCs and cell cultures

iDCs were generated from human CD14+ monocytes obtained from leukapheresis of healthy donors and treated with IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) for 5 days as described.[27] After 5 days, cells had an iDC phenotype: CD14−, CD1a++, CD86+, HLA-DR++. In some experiments iDCs were seeded at 1.2×10^6 to 2×10^6 cells per 2 mL in 12-well plates in RPMI 1640 with 5% FBS with or without graded doses of *Escherichia coli* LPS (0.25 to 1 μg/mL) (Sigma, St Louis, MO) alone or with graded amounts of NOR4 (25 to 100 μM) (Calbiochem, San Diego, CA) or the vehicle DMSO (Sigma). In other experiments the cGMP analog, 8-bromo-cGMP (8B-cGMP), (Sigma) was used to mimic an increase in cGMP levels. The highly specific cGK inhibitor DT-3 (Calbiochem), a membrane-permeable peptide that is 20 000 times more selective for cGK (K_i = 25 nM) than cAK (K_i = 493 μM),[28] was also used. In some experiments we used a specific cAK inhibitor derived from the endogenous protein kinase A inhibitor (PKI) consisting of the peptide sequence 14 to 22 of PKI (K_i = 36 nM) that has been myristoylated at the N terminus (myrPKI) to enhance cell permeability (Calbiochem).[29]

Fluorescence-activated cell sorting (FACS) analysis

DCs were stained with the following conjugated monoclonal antibodies (mAbs): CD1a-PE (SFcI19thy1A8) (Beckman Coulter, Miami, FL); CD14-FITC (MΦP9) (BD Bioscience, Lexington, KY); CD86-PE (IT2.2), CD83-FITC (HB15), CXCR4-PE (12G5), CCR5-FITC (2D7/CCR5), CD38-PE (HIT2) (BD Bioscience Pharmingen, San Diego, CA); CD54 (LB2/H616, Clark Laboratory, Seattle, WA). CCR7 was detected using mouse anti–human CCR7 (2H4) (BD Bioscience Pharmingen), followed by FITC-labeled rabbit antimouse specific for IgG and IgM (BioSource, Camarillo, CA). The intracellular detection of DC-LAMP was performed as described[27] using mouse anti–human DC-LAMP (104.G4) (Immunotech Beckman Coulter, Somerset, NJ). Fluorescence acquisition was done on FACScan analyzer (Becton Dickinson, San Jose, CA) and data analysis with CellQuest software.

Cytokine detection

IL-12p70, IL-6, TNF-α, and IL-10 enzyme-linked immunosorbent assay (ELISA) was performed on supernatants collected from DCs 24 hours after LPS stimulation with or without graded doses of NOR4 or 8B-cGMP. IL-12p70 ELISA (Duo Set; R&D Systems, Minneapolis, MN) was performed according to the manufacturer's instruction. IL-6, TNF-α, and IL-10 were detected by ELISA in triplicate using a matched pair of cytokine-specific mAbs and recombinant cytokines as standards (BD Bioscience Pharmingen): capture anti–IL-6, MQ2-13A5; detection anti–IL-6, MQ2-39C3; capture anti–TNF-α, mAb11; detection anti–TNF-α, mAb11; capture anti–IL-10, JES3-19F1; detection anti–IL-10, JES3-12G8.

Migration assay

DC migration was measured in duplicate using a transwell system (24-well plates; 8.0-μm pore size; Costar, Corning, NY). A total of 600 μL RPMI medium with or without 200 ng/mL recombinant human CCL19/MIP-3β or CXCL12/SDF-1α (RDI, Flanders, NJ) was added to the lower chamber. Wells with medium only were used as a control for spontaneous migration. A total of 2.5×10^5 cells in 100 μL were added to the upper chamber and incubated at 37°C for 3 hours. Cells that migrated into the lower chamber

were harvested, concentrated to a volume of 200 μL, and counted by flow cytometry acquiring events for a fixed time of 30 seconds. The counts fell within a linear range of the control titration curves obtained by testing increasing concentrations of cells. The mean number of spontaneously migrated cells was subtracted from the total number of cells that migrated in response to the chemokine. Values are given as the mean number of migrated cells plus or minus SEM.

Western blotting

iDCs were treated for 24 hours in the presence or absence 0.3 μg/mL LPS with or without 100 μM NOR4, and then 2×10^6 cells were stimulated or not for 2 or 15 minutes with 250 ng/mL human CCL19 or CXCL12 at 37°C. The reaction was stopped with ice-cold PBS, and then cells were spun down at 17 210g for 5 minutes at 4°C and lysed by sonication in lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 2 mM EDTA,1 mM PMSF, 1% Triton X-100) with a protease inhibitor cocktail (Sigma). Proteins were assayed by BCA assay (Pierce, Rockford, IL). Lysates were resolved by an 8% to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel and electrotransferred to PVDF membranes. Immunoblotting was performed with the monoclonal anti–phospho-VASP (Ser239) antibody (16C2) (Alexis, San Diego, CA).[24] Normalization of phosphorylated VASP was performed either with the polyclonal anti-VASP (M4) (Alexis) or anti-p38 MAPK (C20) (Santa Cruz Biotechnology, Santa Cruz, CA). HRP-conjugated secondary antibodies were used (Jackson ImmunoResearch Labs, West Grove, PA); blots were developed with enhanced chemiluminescence (ECL) and analyzed with Image J 1.33u (National Institutes of Health [NIH], Bethesda, MD) software.

Measurement of intracellular cGMP

iDCs (2.5×10^6 cells) were incubated at 37°C for various periods (30 minutes, 1.5 hours, 5 hours, 24 hours) in the presence or absence of 0.3 μg/mL LPS with or without 100 μM NOR4. Reactions were terminated by adding ice-cold ethanol: 1N HCl, 99:1; lysates were stored at −70°C until cGMP determination. cGMP levels were determined by using the acetylation protocol cGMP EIA kit (American Qualex, San Clemente, CA) according to the manufacturer's instruction. cGMP levels were normalized by cell number.

RNA purification and real-time PCR

Total RNA from iDCs treated for 24 hours in the presence or absence of 0.3 μg/mL LPS with or without 30 to 100 μM NOR4 was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription (RT) of RNA samples (1 μg) was performed using oligo(dT)$_{20}$ and the cloned AMV first-strand cDNA synthesis kit (Invitrogen). Specific primers for human cGKI, Iα, Iβ, and II (primers in Table S1; see the Supplemental Materials link at the top of the online article, at the *Blood* website) were used for quantitative real-time polymerase chain reaction (PCR) amplification in an ABI PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix. Nonspecific amplification was excluded by performing a dissociation curve analysis and by analyzing real-time polymerase chain reaction (PCR) products on 3% agarose gels. Controls were included to exclude amplification of contaminating genomic DNA. cDNA extracted from lung for cGKI and from kidney for cGKII was used as positive controls. The relative expression of the target gene in different samples was normalized to the endogenous β-actin (primers in Table S1) and was calculated with the $2^{-\Delta\Delta C_T}$ method.[30]

Results

NO reduces LPS-induced IL-12 production but does not block DC maturation

To test if increasing NO concentration affects maturation and migration of iDCs, we used NOR4, an NO donor that releases NO at a slow rate,[31] thereby more closely mimicking the production of

NO from cells in inflammatory sites. The maximal concentration of NOR4 used in this study (100 μM) produces NO in the range of plasma concentrations of NO during inflammation.[32] At all concentrations used, NOR4 did not affect DC numbers and viability as measured by trypan blue exclusion and annexin V/PI staining (data not shown). Furthermore, NOR4 had no major effect on LPS-induced DC maturation, including little or no effect on induced expression of either DC-LAMP or CD83 or up-regulation of CD86 (Figure 1A). The adhesion molecule CD54 and the cell surface ectoenzyme CD38, which are up-regulated by LPS and play a role in DC migration,[33,34] also were not substantially affected by the presence of NOR4 during DC stimulation with LPS (Figure 1B and data not shown).

The activation of the cAMP/cAK pathway in DCs (eg, through PGE2 or ATP) drives DCs toward a Th2 immune response via down-regulation of IL-12p70 production. We tested if NO had a similar effect. The addition of either NOR4 or 8B-cGMP in a dose-dependent fashion consistently inhibited LPS-induced production of IL-12p70 (Figure 1C) and TNF-α (Figure S1 and data not shown) without significantly affecting IL-6 production (Figures 1C and S1 and data not shown). NOR4 also reduced LPS-induced increases in IL-10 about 2-fold ($P < .02$; Figure S1). Therefore, while NO does not significantly affect DC maturation induced by LPS, it does selectively decrease the release of a set of cytokines produced by DCs.
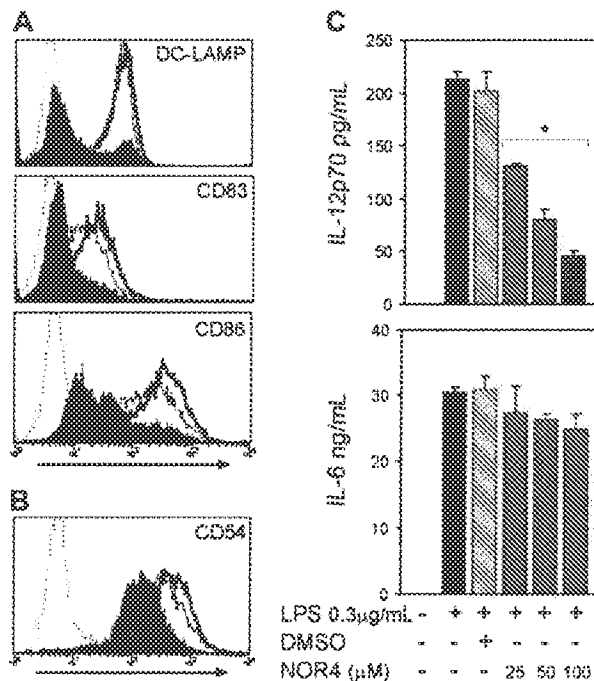

Figure 1. NO reduces the LPS-induced release of IL-12p70 without affecting DC maturation. (A-B) Intracellular expression of DC-LAMP and surface expression of CD83 and CD86 (A) and CD54 (B) in DCs matured for 24 hours with 0.3 μg/mL LPS only (bold open histograms) or plus 100 μM NOR4 (open histograms). iDCs treated for 24 hours in medium only are represented by filled histograms. Isotype controls are indicated by dotted histograms. Data shown are representative of more than 10 independent experiments for DC-LAMP, CD83, and CD86 and 3 experiments for CD54. (C) iDCs were stimulated or not (□) for 24 hours with LPS (0.3 μg/mL) in the presence (▨) or absence (▨) of graded doses of NOR4 (25, 50, 100 μM) or the vehicle control DMSO (▨). Supernatants were collected and analyzed for IL-12p70 and IL-6 by ELISA. *$P < .001$ compared with control LPS-treated DCs (▨), unpaired Student t test. Data are from 1 representative experiment of 9 independent experiments performed using cells from different donors. A statistical analysis of the 9 experiments using the paired t test gave a value of $P = .01$, confirming that the inhibition of IL-12p70 production by NOR4 is statistically significant.
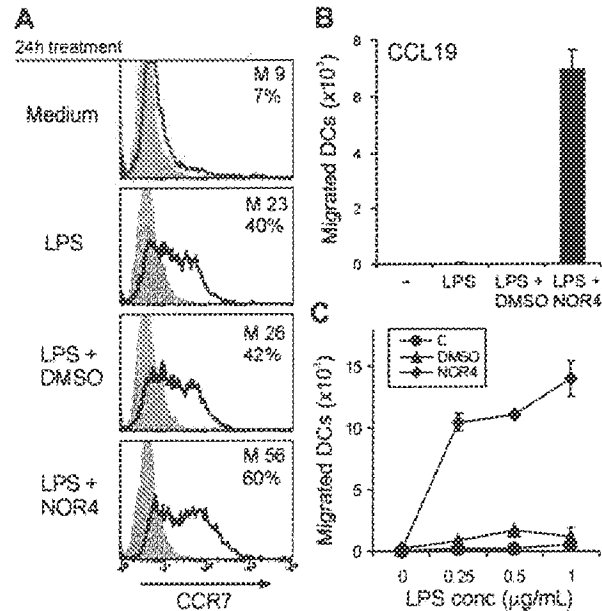

Figure 2. NO induces DC migration toward CCL19/MIP-3β. (A) Surface CCR7 expression of iDCs matured or not for 24 hours with LPS (0.3 μg/mL) in the presence or absence of 100 μM NOR4 or DMSO. The mean fluorescence intensity (M) and percentages of positive cells (%) are indicated. Data shown are representative of more than 10 independent experiments. (B) iDCs matured or not for 24 hours with 0.3 μg/mL LPS in the presence or absence of 100 μM NOR4 or DMSO were tested for their migration toward CCL19. Data shown are mean of duplicate cultures ± SEM and are representative of 9 independent experiments. (C) iDCs were matured for 24 hours with graded doses of LPS with medium only (●), 100 μM NOR4 (♦), or 0.001% DMSO (▲) and tested for migration toward CCL19. Data are from 1 representative experiment of 2 performed using cells from different donors.

NOR4 induces DC migration of LPS-matured DCs toward CCL19/MIP-3β

We next tested if NO affects migration of LPS-matured DCs toward CCL19/MIP-3β, as shown for other inflammatory factors.[13,14] LPS alone induced a substantial increase in CCR7 expression (Figure 2A) but did not induce DCs to be responsive to CCL19 (Figure 2B). The addition of 100 μM NOR4 with LPS potently enabled DCs to respond to CCL19 and migrate (Figure 2B), and the effect of NOR4 was dose dependent (Figure 3B). LPS-induced up-regulation of CCR7 was only slightly enhanced by NOR4 (Figure 2A). Thus, NOR4-induced enhancement of migration toward CCL19 was not simply due to an increase in CCR7. The NOR4 effect on migration increased with graded doses of LPS (Figure 2C), suggesting a synergistic effect between NOR4 and LPS in sensitizing DCs to migrate. A substantial effect of NOR4 was evident with as little as 0.3 μg/mL LPS, a concentration that induces complete maturation of iDCs. Therefore, we used this concentration of LPS in the following experiments unless indicated otherwise.

We tested if NO could increase DC migration not only to CCL19 but also to other chemokines. Upon maturation iDCs down-regulate CCR5 and up-regulate CXCR4, a receptor for CXCL12/SDF-1α, a chemokine that regulates migration of DCs toward LNs.[35] NOR4 enhanced LPS-induced down-regulation of CCR5 (data not shown) and up-regulation of CXCR4 in a dose-dependent fashion (Figure 3). LPS-treated DCs were already responsive to CXCL12; NOR4 also enhanced LPS-induced migration toward CXCL12 (Figure 3B). In this case the NOR4-dependent enhancement of migration toward CXCL12 was gradual as well as the increase in CXCR4 expression (Figure 3). In contrast, maximal up-regulation of CCR7 was evident at the lower dose of
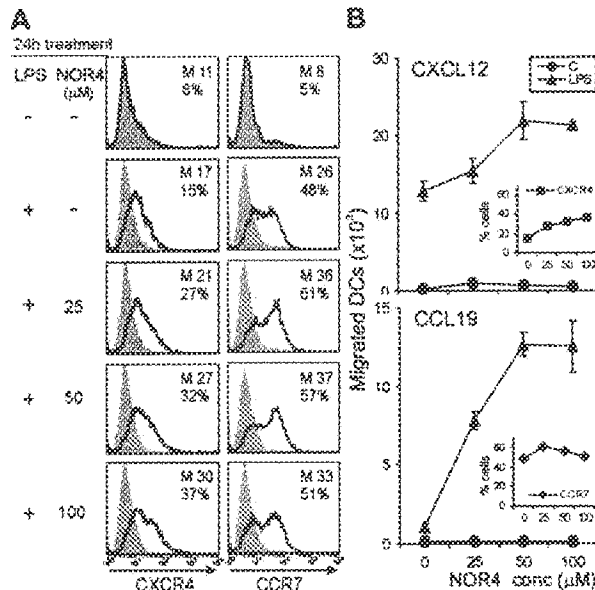
Figure 3. NO enhances LPS-induced expression of CXCR4 and migration toward CXCL12/SDF-1α. iDCs were treated or not for 24 hours with 0.3 µg/mL LPS in the absence or presence of graded doses of NOR4. (A) Surface expression of CXCR4 or CCR7. The mean fluorescence intensity (M) and percentages of positive cells (%) are indicated. (B) Cells treated with medium only (●) or 0.3 µg/mL LPS plus graded doses of NOR4 (▲) were tested for their chemotactic response toward CXCL12 or CCL19. The receptor levels after stimulation with LPS plus graded doses of NOR4 are shown in the inserts; the percentages of cells expressing CXCR4 (■) and CCR7 (◆) are shown in the top and bottom panels, respectively. Data shown are representative of 3 independent experiments.

NOR4, while optimal migration toward CCL19 was only evident at higher doses (Figure 3). These data suggest that NOR4 promotes increased DC migration to CCL19 via an additional mechanism independent from altering receptor expression. NOR4 affected specifically chemokine-directed chemotaxis and had little or no effect on spontaneous migration in the absence of chemokines (data not shown). Furthermore, treatment of iDCs with graded doses of NOR4 alone did not induce increases in CCR7 or CXCR4 expression (not shown) or promote migration toward CCL19 or CXCL12 (Figure 3B). Therefore, the enhancement of DC migration by NO requires a maturation stimulus.

Moreover, NOR4 also enhanced the reciprocal regulation of CCR5 and CXCR4 induced by CD40 stimulation (D.G. and L.A.C., unpublished data, March 2005), suggesting that NO regulates chemokine receptor levels irrespective of the maturation stimulus.

The effects of NOR4 on both chemokine receptor expression and migration toward CCL19 and CXCL12 suggest that elevated levels of NO in inflammatory sites may drive DCs toward a more mature phenotype and facilitate migration of DCs toward LNs.

Role of cGMP and cGK in NO-regulated DC migration

Because NO acts mainly through the activation of the cGMP/cGK pathway, we next tested if DC migration toward either CCL19 or CXCL12 could be enhanced by the cell-permeable analog, 8B-cGMP. Unlike NOR4, which enhanced migration of LPS-treated DCs toward CCL19 at relatively low concentrations, 8B-cGMP affected DC migration only at high concentrations (Figure 4A). Although a modest increase in cGMP levels appeared to be sufficient to enhance CCR7 expression, enhanced migration toward CCL19 apparently requires a high intracellular level of cGMP

and/or a cGMP-independent mechanism. Low concentrations of 8B-cGMP (300 µM) enhanced DC migration toward CXCL12 (Figure 4B), suggesting that smaller increases in cGMP are needed to enhance CXCL12- versus CCL19-dependent migration.

To further analyze the role of the cGMP/cGK pathway, we tested if a highly specific cGK inhibitor, the cell-permeable peptide DT-3,[28] could affect NOR4-dependent DC migration. If NOR4 enhances migration mainly via activation of the cGMP/cGK pathway, then inhibition of cGK activity by DT-3 should inhibit migration. However, the cGK inhibitor was unable to block NOR4-induced DC migration toward CCL19 and only partially inhibited the enhanced DC migration toward CXCL12 (Figure 4C). Thus, while cGMP may contribute to the increased sensitivity to migration toward CCL19 induced by NOR4, a cGMP/cGK-independent pathway is also involved. Furthermore, the relative contribution of the cGMP/cGK pathway on DC migration seems to be different for CXCL12 versus CCL19.

cGK and cAK regulate the ability of LPS-treated DCs to migrate toward CCL19

We next tested if the cGMP/cGK pathway can directly modulate the chemokine signaling pathways activated through CCR7 or CXCR4. We added the specific cGK inhibitor DT-3 to DCs during the migration assay and, to our surprise, we found that preincubation of LPS-matured DCs with graded doses of DT-3 just prior the chemotactic assay sensitized DCs so they could migrate toward
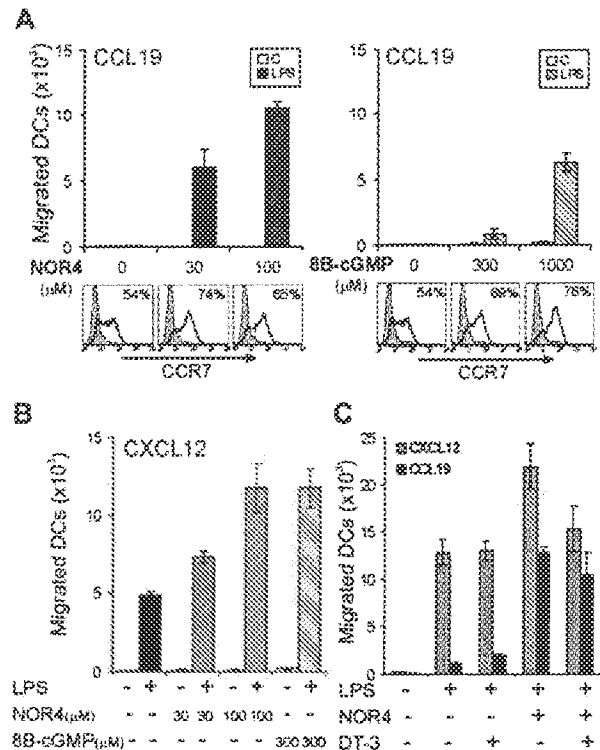

Figure 4. NO induction of migration toward CCL19 is only partially dependent on the cGMP/cGK pathway. (A-B) iDCs were treated or not for 24 hours with 0.3 µg/mL LPS in the presence or absence of graded doses of NOR4 or 8B-cGMP. Surface expression of CCR7, percentages of positive cells (%) are indicated. Chemotactic responses toward CCL19 (A) or CXCL12 (B) are shown as the mean of duplicate cultures ± SEM. Data shown are representative of 3 independent experiments. (C) iDCs were treated or not for 24 hours with 0.3 µg/mL LPS in the presence or absence of 100 µM NOR4 with or without 500 nM DT-3 (cGK inhibitor) and tested for their chemotactic response toward CCL19 or CXCL12. Data shown are representative of 3 independent experiments.
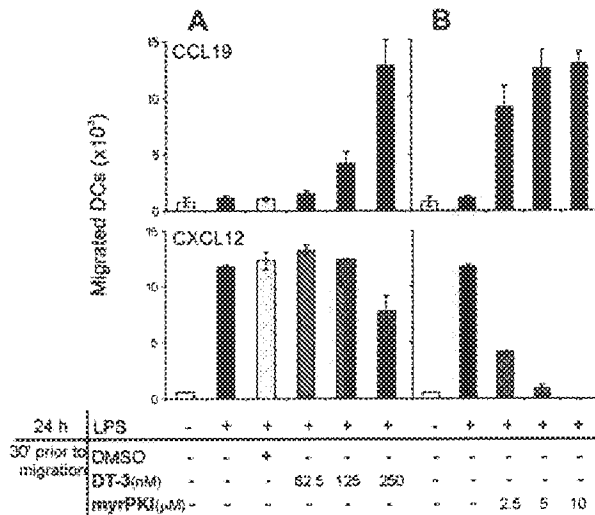
Figure 5. Inhibition of either cGK or cAK allows LPS-matured DCs to migrate toward CCL19, while migration toward CXCL12 requires cAK activity. iDCs were treated (■) or not (□) with 0.3 µg/mL LPS for 24 hours. Cells were harvested, incubated with graded doses of either the cGK inhibitor DT-3 (A) or the cAK inhibitor myrPKI (B), and then tested for their chemotactic response toward CCL19 or CXCL12. Data shown are representative of 3 experiments using different donors.

CCL19 (Figure 5A, top). Therefore, persistent cGK activity prevents mature DCs from migrating toward CCL19. These data again suggested that NO affects DC migration via a mechanism independent from the "activation" of the cGMP/cGK pathway. DT-3 had little or no effect on LPS-induced migration toward CXCL12 (Figure 5A, bottom). These data further support the hypothesis that the cGMP/cGK pathway plays a different role in regulating CCL19 versus CXCL12 migration.

Because NO also can activate the cAMP signaling pathway,[36] we tested if a specific cAK inhibitor, permeable peptide myristoylated PKI (myrPKI), could affect DC migration. As observed for cGK inhibition, inhibition of cAK during the chemotactic assay also enabled LPS-treated DCs to migrate toward CCL19 (Figure 5B, top). However, LPS-induced sensitivity to migration toward

CXCL12 was completely blocked by the cAK inhibitor (Figure 5B, bottom). The addition of DT-3 or myrPKI in the chemotactic assay did not affect chemokine-specific chemotaxis of DCs not treated with LPS, and random cell motility was not significantly affected in either immature or LPS-treated DCs (data not shown). Taken together these data suggest a differential regulation by cyclic nucleotides of DC migration toward CCL19 versus CXCL12. Both cGK and cAK activities potently inhibit migration signals induced by CCL19 interaction with CCR7. In contrast, cAK activity, but not cGK activity, is required to allow DC migration toward CXCL12.

NO reduces cGK-mediated phosphorylation of VASP, which may prevent DCs from migrating toward CCL19

The data reported in the previous section suggest that cGK and cAK activities already expressed or induced by LPS prevent DCs from migrating toward CCL19. Thus, we reconsidered the possible mechanisms by which NOR4 facilitates the migration of LPS-triggered DCs (Figure 4A). The cGMP/cGK pathway appeared to be able to exert either a positive (as shown earlier) or a negative effect on DC migration. How might this occur? One possibility was that after the initial activation of the cGMP/cGK pathway by NOR4, long-term treatment with the NO donor reduces cGK activity so that in the absence of cGK a brake is released enabling DCs to migrate.

To test this hypothesis, we first examined if NO could reduce cGK activity constitutively expressed or induced after LPS treatment. To measure cGK activity, we selected the cGK target VASP, because VASP is involved in cytoskeletal rearrangements occurring during cell migration.[26] Interestingly, cGK-dependent VASP phosphorylation induces detachment of VASP and other cytoskeletal proteins from focal adhesions, thus inhibiting cell migration.[26,37] Thus, phosphorylated VASP might contribute to the inhibitory effect of cGK activity on DC migration. We measured cGK activity by using an mAb (VASP-16C2) that specifically detects phosphorylation of VASP on serine 239 phosphorylation, an established and useful monitor of cGK activity in intact cells.[24,37] The basal level of cGK activity in iDCs was very low, but LPS stimulation alone
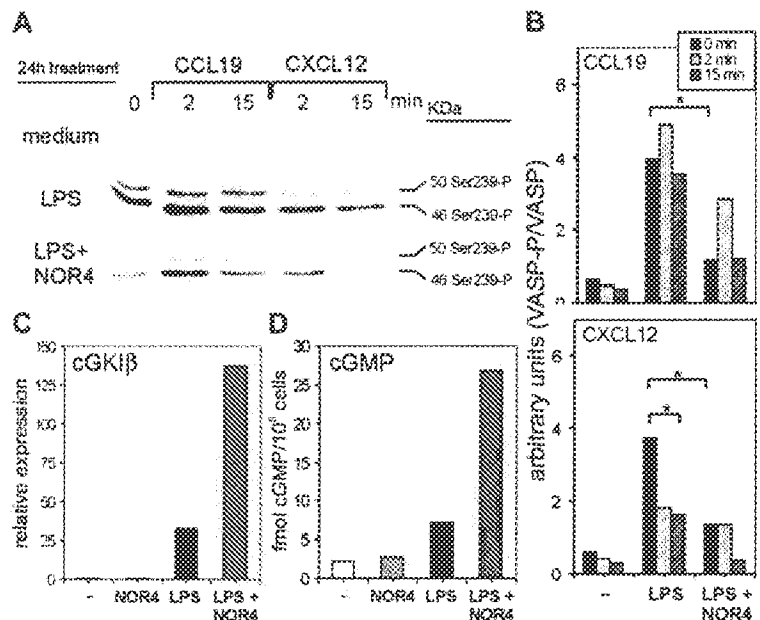

Figure 6. LPS induces cGK-dependent phosphorylation of VASP in DCs, which is inhibited by NOR4. iDCs were treated or not for 24 hours with 0.3 µg/mL LPS in the presence or absence of 100 µM NOR4. Cells from each group were then stimulated for the indicated times with either CCL19 or CXCL12. (A,B) Cell extracts were prepared and immunoblotted with anti–phospho-VASP antibody and then with anti-VASP antibody. (A) Antiphospho-VASP immunoblot of 1 representative experiment of 3 performed with cells from different donors. (B) Quantitative densitometric analysis of 3 different immunoblot experiments. The activated phospho-VASP expression was normalized to the total VASP. *P < .001, paired Student t test. (C) mRNA levels of cGKIβ determined by quantitative real-time RT-PCR. The relative expression of cGKIβ was normalized to the endogenous β-actin. Data shown are from 1 representative of 2 independent experiments giving the same results. (D) cGMP levels in cell extracts were measured with cGMP EIA assay. Data shown are representative of 2 independent experiments.

induced a substantial increase in cGK-dependent VASP phosphorylation (Figure 6A). Thus, cGK activity is not constitutively expressed in DCs but is up-regulated by LPS. The addition of NOR4 in combination with LPS strongly inhibited VASP phosphorylation (Figure 6A, first lane, and Figure 6B), indicating that NOR4 blocks the increase in cGK activity induced by LPS. The overall levels of VASP protein did not change in any of the DC groups (Figure 6B and data not shown). Thus, the observed changes in VASP phosphorylation were not due to changes in expression of total VASP.

The experiments using the cGK inhibitors suggested that cGK activity might be differently affected by CCR7 versus CXCR4 signaling. To test this, we compared cGK-dependent VASP phosphorylation after stimulating iDCs, LPS-matured DCs, or LPS plus NOR4-matured DCs with CCL19 or CXCL12 (Figure 6A). Cells treated only with LPS upon CCL19 stimulation retained a high level of VASP phosphorylation, consistent with their inability to migrate toward CCL19 (Figures 2, 3, and 6A-B). Contrary to CCL19 stimulation, CXCL12 stimulation of LPS-treated DCs decreased VASP phosphorylation, again consistent with the ability of DCs to migrate toward CXCL12 (Figures 3 and 6A-B). For both chemokines NOR4 treatment during LPS maturation further reduced the level of VASP phosphorylation after stimulation, consistent with the ability of NO to induce migration toward CCL19 and enhance migration toward CXCL12 (Figure 6A-B).

How might long-term treatment with NOR4 reduce cGK activity in LPS-treated DCs? One possibility was that exposure to NO reduces cGK expression in DCs, as reported in other cell types.[38,39] To determine which cGK isoforms are expressed in DCs, we used real-time PCR. Mammals have 2 cGK genes: one encodes for the soluble form cGKI, which produces 2 alternatively spliced isoforms, cGKIα and cGKIβ; the other encodes for membrane-associated cGKII.[40] We detected almost no cGKI and very low levels of cGKII expression in iDCs (Figures 6C and S2), and cGKII expression was not significantly affected by any treatment (Figure S2). However, LPS induced a large increase in cGKI at 24 hours (Figures 6C and S2) that was entirely due to increases in cGKIβ (Figure S2). This induction of cGKIβ expression was consistent with the increased cGK-dependent VASP phosphorylation observed after LPS treatment. NOR4 alone had no effect on cGKI but together with LPS induced 4- to 5-fold increases in cGKI levels in DCs compared with LPS treatment only (Figures 6C and S2). Therefore, the observed decrease in cGK-dependent VASP phosphorylation induced by NOR4 was not due to a down-regulation in cGK mRNA expression.

A reduction in cGK activity after NOR4 treatment could also be mediated by a decrease in cGMP levels caused by either the down-regulation of guanylate cyclases, the cGMP-synthesizing enzymes, or by the up-regulation of cGMP-phosphodiesterases, the hydrolyzing enzymes. Similarly to cGK, an NO-induced long-term desensitization has also been described for guanylate cyclases.[41] To test if long-term treatment of NOR4 down-regulates cGMP in DCs we examined changes in cGMP levels during treatment of DCs with LPS alone or with NOR4. Within 30 to 60 minutes NOR4 induced an initial increase in cGMP in DCs treated or not with LPS (Figure S3). After this initial peak cGMP levels decreased in DCs treated only with NOR4 but were sustained in DCs treated with both LPS and NOR4 (Figure S3), reaching 20-fold higher levels than DCs treated only with LPS for 24 hours (Figure 6D). Therefore, long-term treatment of DCs with LPS plus NOR4 leads to continuous release of NO and consequently a sustained increase

in cGMP levels. NOR4 ability to reduce cGK-dependent VASP phosphorylation induced by LPS cannot be due to a decrease in cGMP levels.

Overall our data suggest that LPS increases both cGK expression and activity in DCs, leading to phosphorylation of VASP, which is in part responsible for preventing DCs from migrating toward CCL19. Long-term NOR4 treatment inhibits the increase in cGK-dependent phosphorylation of VASP, thereby releasing a brake so that DCs can migrate.

Discussion

NO regulates migration of human DCs toward the LN-directing chemokines CCL19 and CXCL12. Whereas LPS-stimulated monocyte-derived DCs do not migrate toward CCL19, NOR4 enables them to migrate (Figure 2B). NOR4 enhanced LPS-induced expression of the CCR7 and CXCR4 receptors; however, enhancement by NO of chemokine receptor expression alone could not account for why DCs were able to migrate. The effects of NO on DC chemokine receptor expression and migration parallel the effects of cAMP-increasing inflammatory stimuli;[10-13] both stimuli couple CCR7 to signal transduction pathways that allow migration, thus sensitizing DCs to migrate toward CCL19.

While chemokine receptor expression can be uncoupled from the ability of DCs to migrate,[41,42] the mechanism responsible for this has not been elucidated. Surprisingly, the inhibition of cGK prior to the chemotactic assay enabled DCs to migrate toward CCL19 (Figure 5), suggesting that cGK activity constitutively expressed or induced by LPS might prevent DCs from migrating even though they express chemokine receptors. Consistent with this model, LPS induced DCs to express cGKI (Figure 6C) and sustained cGK activity as measured by phosphorylation of the key cGK target VASP (Figure 6A). VASP expression and regulation upon chemokine stimulation has not been previously reported in DCs. Besides being a useful tool to monitor cGK activity,[24] VASP may also provide a possible explanation for the inhibitory role of cGK in DC migration. VASP binds cytoskeletal proteins zyxin and vinculin involved in focal adhesion formation; cGK-dependent VASP phosphorylation results in depletion of these cytoskeletal proteins from focal adhesions, destabilization of motility structures, and inhibition of migration.[26,27] The substantial increase in cGK-dependent VASP phosphorylation induced by LPS was retained upon short-term CCR7 stimulation with CCL19. Thus, phosphorylation of VASP correlated with unresponsiveness to CCL19. High cGK activity and subsequent VASP phosphorylation may disrupt focal adhesion formation and DC migration (Figure 7).

VASP may not be the only cGK target in LPS-stimulated DCs. Two actin binding proteins, LIM and LASP-1, are direct substrates for cGK and cAK; upon phosphorylation their affinity for F-actin is reduced, causing their relocalization from membrane extensions to the cytosol and reducing cell migration.[44,45] Furthermore, cAK- and cGK-dependent VASP phosphorylation can inhibit integrin $\alpha_{IIb}\beta_3$ activation, another important step for cell polarization and migration.[46]

The presence of NOR4 during DC maturation strongly reduced basal levels of cGK-dependent VASP phosphorylation, and this inhibition was maintained after either CCL19 or CXCL12 stimulation (Figure 6A-B). The absence of high levels of phosphorylated

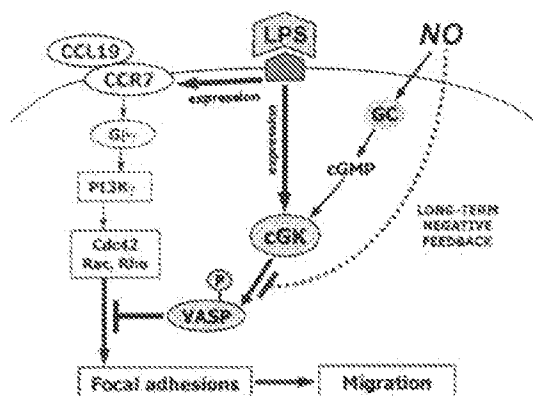From www.bloodjournal.org by on December 10, 2009. For personal use only.

Figure 7. NO promotes LPS-mediated DC migration toward CCL19. LPS alone induces expression of cGK and phosphorylation of VASP, thereby preventing DCs from responding to CCL19 and migrating. NO releases DCs from this inhibition via a feedback inhibition pathway that blocks cGK-dependent phosphorylation of VASP.

VASP correlated with the ability of DCs to migrate. Thus, NO may sensitize DCs so they can migrate through the inhibition of LPS-induced increases in cGK activity and VASP phosphorylation (Figure 7). cGK-associated signaling pathways are controlled by feedback regulation.[41,47] One feedback regulatory mechanism is the suppression of cGKIα expression by long-term exposure to NO-releasing agents.[38,39] However, NOR4 did not reduce cGK activity in DCs simply by inhibiting LPS-induced cGK expression, which was rather enhanced by NOR4 (Figure 6C). Also, DCs treated with both LPS and NOR4 had higher cGMP levels than DCs treated with only LPS. Thus, the lower cGK activity in the NOR4 plus LPS-treated cells was not due to decreased production or increased degradation of cGMP, although the possibility of reduced cGMP in cell microcompartments cannot be excluded. After the initial activation of the cGMP/cGK pathway, long-term treatment with NO may induce a negative feedback pathway that reduces cGK-dependent VASP phosphorylation in DCs (Figure 7). It remains to be determined whether this reduction is due to posttranslational regulation, a direct inhibition of cGK activity or another mechanism. Although NO acts mainly through the cGMP pathway, it can also directly modify lipids, nucleic acids, and proteins. For instance, the activity of many transcriptional factors or cysteine proteases is altered by direct nitrosylation from NO.[48] We are currently investigating the mechanisms responsible for the reduced cGK activity induced by NOR4 in DCs.

NO also enhanced the migration of LPS-treated DCs toward CXCL12; consistent with some findings[42] but in contrast to others,[14] matured DCs were sensitive to CXCL12-dependent migration even in the absence of NO or other signals (Figure 3B). In contrast to CCL19 stimulation, short-term stimulation of LPS-treated DCs with CXCL12 reduced cGK-dependent VASP phosphorylation (Figure 6A-B), consistent with the ability of the DCs to migrate toward CXCL12. One explanation for the differential sensitivity of LPS-treated DCs to migrate toward CCL19 versus CXCL12 may be that CXCL12 alone, unlike CCL19, can directly reduce VASP phosphorylation and release DCs to migrate. The cAK inhibitor myrPKI selectively blocked DC chemotaxis toward CXCL12 but not to CCL19, underscoring another difference between CXCR4 and CCR7 signaling pathways: cAK activation is required for CXCL12-dependent migration but not for CCL19-dependent migration. Ligation of CXCR4 by CXCL12 activates the cAMP/cAK pathway in T cells.[38]

Cell migration is a complex phenomenon that involves cell polarization and requires a dynamic regulation of receptors, adhesion molecules, cytoskeletal proteins, and intracellular regulatory molecules, with coordinated formation and degradation of gradients of second messengers, like calcium or cAMP.[51] These requirements may explain why the NO and cGMP/cGK pathway has been implicated in both positive and negative effects on cell migration. The activation of the cGMP/cGK pathway by NO positively regulates migration, possibly through the induction of chemokine receptor expression[41] (Figure 4A). However, we also found that high levels of cGK activity in LPS-stimulated DCs may inhibit migration (Figures 5 and 6A-B). In addition to its role in VASP regulation, the cGK pathway can negatively affect migration in other ways. NO, via cGMP/cGK, inhibits smooth muscle cell migration by either activating a MAPK phosphatase, which inactivates MAPKs,[52] or by activating a tyrosine phosphatase that decreases tyrosine phosphorylation of proteins involved in focal adhesion formation and actin polymerization.[53,54] cGK reduces myosin light chain (MLC) phosphorylation, an important step for cell polarization and migration, either by phosphorylating and decreasing MLC kinase activity or by activating MLC phosphatase.[55,56] Another major target of cGK is IP₃ R-associated cGK substrate (IRAG), which when phosphorylated inhibits IP₃-induced calcium release from intracellular stores.[57] Thus, cGK may block another step in the signaling activated by chemokine receptor engagements, because DC migration toward CCL19 is dependent on CCL19-induced intracellular calcium mobilization.[58] Further studies are required to determine the various mechanisms by which NO and the cGMP pathway may regulate DC movement.

This study is the first to define cGK expression and activity in DCs and the important role cGK plays in regulating DC migration toward LN-directing chemokines. An interesting possibility is that a negative feedback mechanism similar to that reported here for NO might be responsible for the observed effect of PGE2 on DC migration. Consistent with this possibility, we found that cAK sensitizes DCs to migrate toward CCL19 as well as cGK. Scandella et al found that PGE2 can induce a marked reduction in the expression of the PGE2 receptors EP2 and EP4 [13] as one possible means of feedback control.

NOR4 did not affect the induction of DC maturation by LPS but did affect cytokine production by DCs. NO inhibited IL-12 p70, IL-10, and TNF-α production by mature DCs but did not affect IL-6 release. Using a protein array system to detect the expression of a range of cytokines and chemokines, we did not detect any other major changes by adding NOR4 to LPS-treated DCs (D.G. and E.A.C., unpublished data, November 2004). These results are consistent with previous work showing that inducible nitric oxide synthase (iNOS)-deficient mice make more IL-12 p40 than wild-type mice in response to LPS[32] and with the recent observation that NO inhibits LPS-induced expression of IL-12 p35 and IL-12 p40 mRNA in murine macrophages and DCs through the inhibition of NF-κB activation.[21] The cGMP analog, 8Br-cGMP, was able to mimic the effect of NOR4, suggesting that the cGMP-dependent pathway is involved in NO-dependent cytokine down-regulation. The cGMP pathway can inhibit NF-κB activation via I-κB stabilization.[41]

Several studies, including studies with iNOS-deficient mice, have implicated NO in the regulation specific immune responses.[18,19,60] NO can inhibit T-cell proliferation, either in a cytokine-dependent or -independent manner.[61-63] Mice deficient for iNOS develop significantly stronger Th1 immune responses and, unlike wild-type mice, are highly susceptible to *Leishmania major* infection.[60] This defect has been

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attributed mainly to the absence of NO acting directly on T cells. However, our data suggest that iNOS deficiency could lead to T-cell skewing because of dysregulation of DCs as well as T cells. Several studies with iNOS-deficient mice and autoimmune models have implicated NO in the control of autoreactive T cells and prevention of the development of autoimmunity.[64-70] The effect of NO on DC migration and cytokine production reported here might contribute to the observed protective effects of NO in autoimmune disorders.

Acknowledgments

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EXHIBIT D
BioCarta- Charting Pathways of Life
(Dendritic cells in regulating Th1 and Th2 Development)
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BIOCARTA

Charting Pathways of Life

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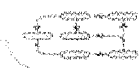
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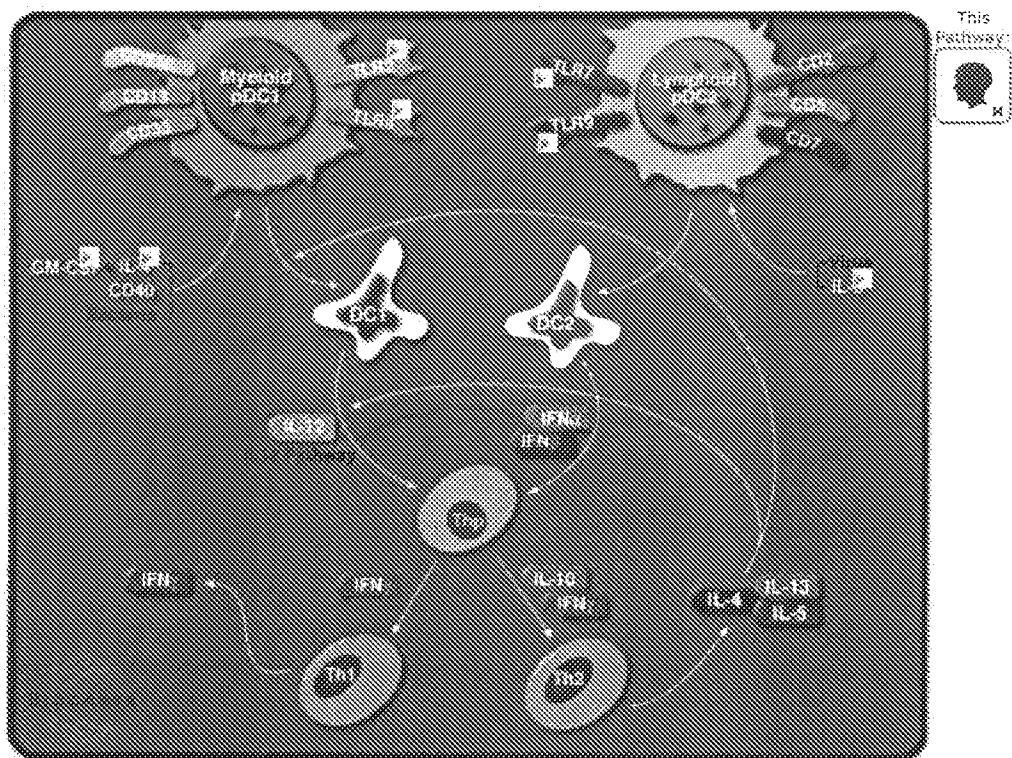
DESCRIPTION: While T cells and B cells carry out the actions of antigen-specific immune responses, antigen-presenting cells called dendritic cells are required for this to happen. The name of dendritic cells is based on their shape, with activated dendritic cells displaying long processes on their surface. When immature dendritic cells found throughout the body internalize and present antigen, they express markers that stimulate the activation of lymphocytes, and migrate to lymphocyte rich tissues like the spleen and lymph nodes to initiate immune responses. In addition to stimulating responses against antigens, dendritic cells also produce tolerance to self antigens.

Dendritic cells can be derived from either myeloid or lymphoid lineages. Monocyte derived lineages (pDC1) stimulate Th1 cell differentiation while plasmacytoid (lymphoid) dendritic cells (pDC2) induce Th2 cell differentiation. Factors that stimulate the maturation of monocytes derived dendritic cells include GM-CSF, and IL-4. IL-3 stimulates the differentiation of pDC2 cells into DC2 cells. A variety of factors are involved in antigen-recognition and processing by immature dendritic cells and in the maturation of these cells. The transition to mature dendritic cells down-regulates the factors involved in antigen internalization, and increases the expression of MHC, costimulatory molecules involved in lymphocyte activation, adhesion molecules, and specific cytokines and chemokines. Toll-like receptors on the surface of immature dendritic cells recognize microbial components to induce dendritic cell maturation (see "Toll-like receptor pathway"). In addition to stimulating B cell responses, dendritic cells are potent activators of T cells. IL-12 secretion by dendritic cells stimulates T cell responses, particularly differentiation of Th1 cells that produce interferon-gamma and other pro-inflammatory cytokines (See "Th1/Th2 Differentiation" pathway). While IL-4 generally stimulates Th2 differentiation, the stimulation of Th2 cell formation by DC2 cells does not appear to involve IL-4. The stimulation of Th1 and Th2 cell formation by dendritic cells appears to be balanced by counter-regulation of each pathway by the other. Interferon-gamma produced by Th1 cells blocks the further stimulation of Th1 differentiation by DC1 cells. The IL-4 produced by Th2 cells kills dendritic cell precursors that contribute to Th2 cell creation. Direct interactions between T cells and dendritic cells are enhanced through the expression of adhesion molecules and costimulatory receptors CD80 and CD86 expressed by mature dendritic cells activate T cells in concert with the recognition of antigen/MHC by the T cell receptor. The central role of dendritic cells as modulators of immune responses makes them an important focus of studies about autoimmune disease, transplant rejection, allergies, responses to infections, and other alterations of the immune response.

CONTRIBUTORS:

REVISION HISTORY:

REFERENCES: Barton GM, Medzhitov R. Control of adaptive immune responses by Toll-like receptors. Curr Opin Immunol 2002 Jun;14(3):380-3

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BioCarta

Charting Pathways of Life
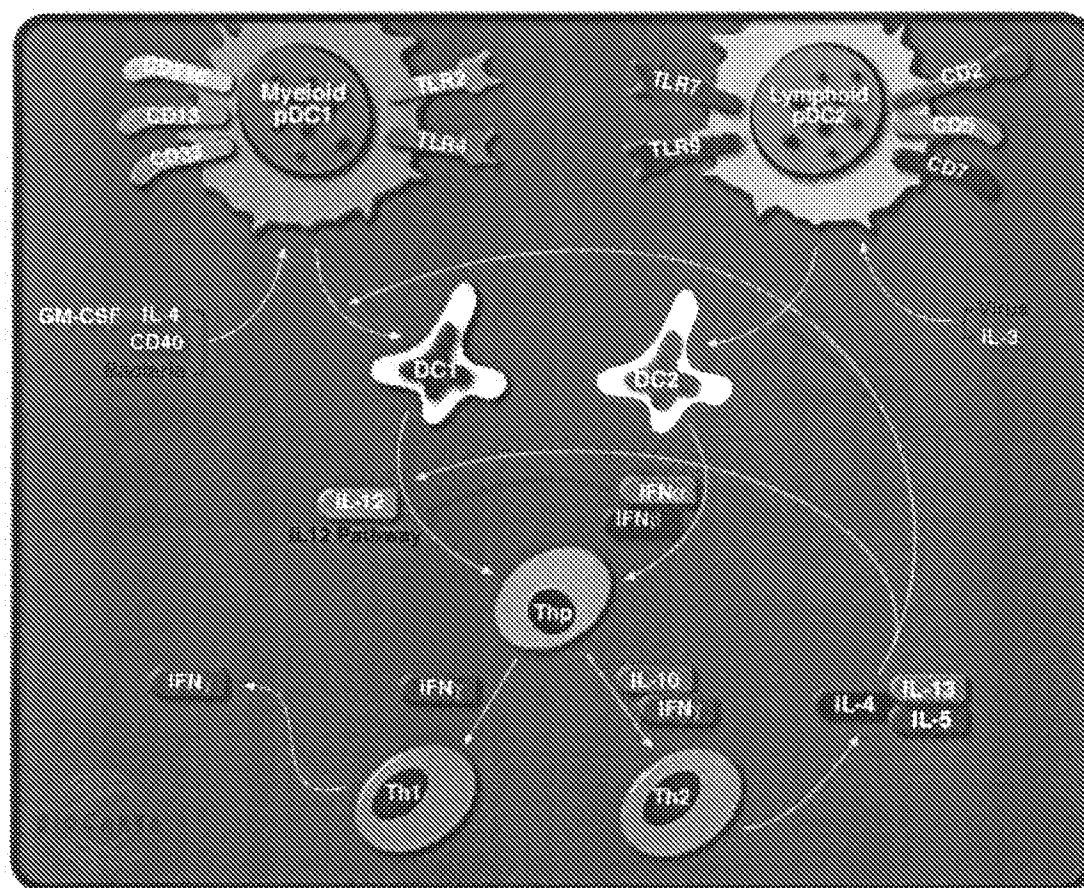
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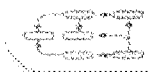
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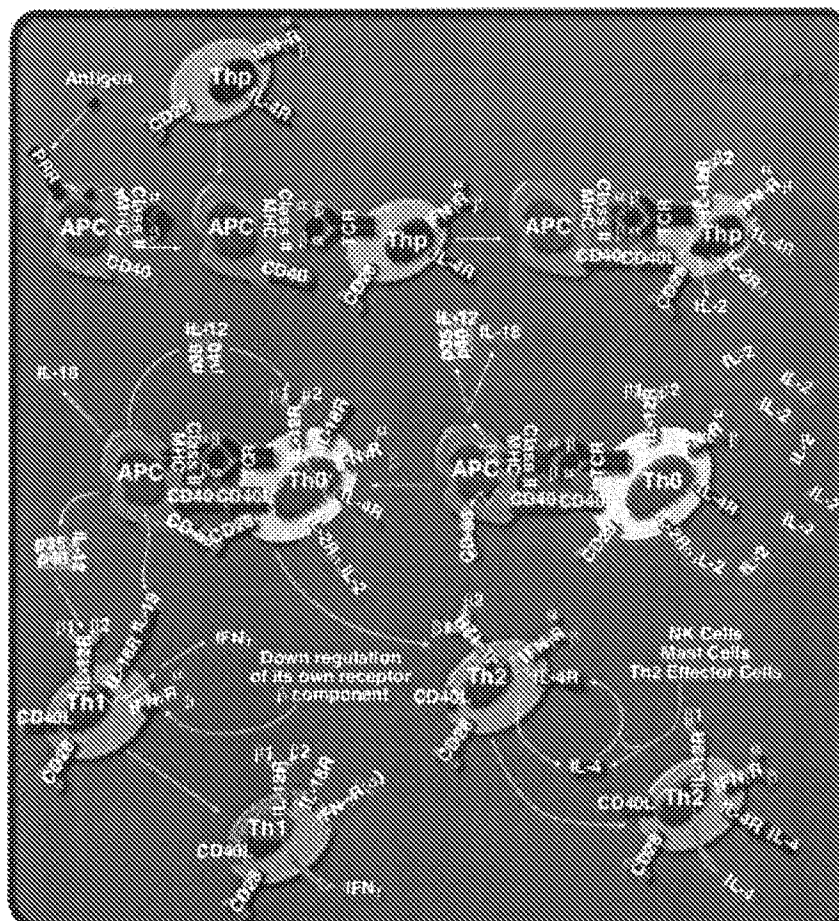
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This Pathway:

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DESCRIPTION: Helper T cells are found in two distinct cell types, Th1 and Th2, distinguished by the cytokines they produce and respond to and the immune responses they are involved in. Th1 cells produce pro-inflammatory cytokines like IFN-g, TNF-b and IL-2, while Th2 cells produce the cytokines IL-4, IL-5, IL-6 and IL-13. The cytokines produced by Th1 cells stimulate the phagocytosis and destruction of microbial pathogens while Th2 cytokines like IL-4 generally stimulate the production of antibodies directed toward large extracellular parasites (see "IL-4 Signaling Pathway"). IL-5 stimulates eosinophil responses, also part of the immune response toward large extracellular parasites (see "IL-5 Signaling Pathway")

Th1 and Th2 are produced by differentiation from a non-antigen exposed precursor cell type, Thp. Exposure of Thp cells to antigen by antigen-presenting cells may result in their differentiation to Th0 cells, not yet committed to become either Th1 or Th2 cells, although the existence of Th0 cells is controversial. Cells committed as either Th1 and Th2 cells are called polarized, whether they are effector cells actively secreting cytokines or are memory cells. The stimulation of Thp cells by exposure to antigen-presenting cells induces the proliferation of undifferentiated cells, and their expression of IL-2 and IL-2 receptor. The differentiation of Th1 cells and Th2 cells depends on the cytokines they are exposed to. IL-12 causes Th1 differentiation and blocks Th2 cell production (see "IL12 and Stat4 Dependent Signaling Pathway in Th1 Development" pathway), while IL-4 causes Th2 differentiation and antagonizes Th1 development. IL-18 also induces Th1 differentiation (See "IL-18 signaling pathway"). Polarized Th1 and Th2 cells also express distinct sets of chemokine receptors that further modify their homing and other cellular responses (see "Selective expression of chemokine receptors during T-cell polarization" pathway). Improved understanding of Th1 and Th2 differentiation will improve our overall understanding of the immune system, its response to infection and aberrant responses that lead to disease.
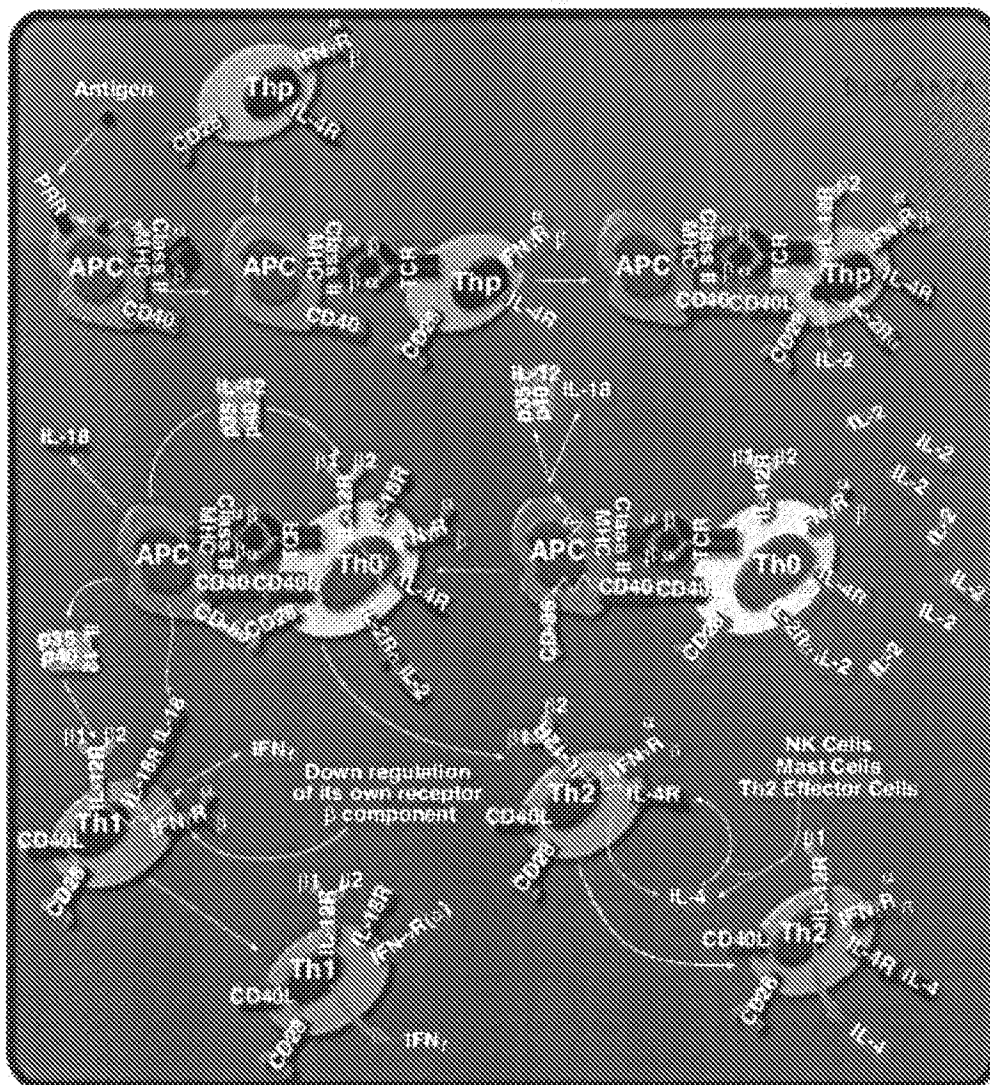
EXHIBIT E
Vieira, *et al.* (2003)
"Glatiramer Acetate (Copolymer-1, Copaxone) Promotes Th2 Cell Development and Increased IL-10 Production through Modulation of Dendritic Cells."
The Journal of Immunology 170: 4483–4488

Glatiramer Acetate (Copolymer-1, Copaxone) Promotes Th2 Cell Development and Increased IL-10 Production Through Modulation of Dendritic Cells[1]

Pedro L. Vieira,[2]* Heleen C. Heystek,* Jan Wormmeester,* Eddy A. Wierenga,* and Martien L. Kapsenberg*[†]

Glatiramer acetate (GA; copolymer-1, Copaxone) suppresses the induction of experimental autoimmune encephalomyelitis and reduces the relapse frequency in relapsing-remitting multiple sclerosis. Although it has become clear that GA induces protective degenerate Th2/IL-10 responses, its precise mode of action remains elusive. Because the cytokine profile of Th cells is often regulated by dendritic cells (DC), we studied the modulatory effects of GA on the T cell regulatory function of human DC. This study shows the novel selective inhibitory effect of GA on the production of DC-derived inflammatory mediators without affecting DC maturation or DC immunostimulatory potential. DC exposed to GA have an impaired capacity to secrete the major Th1 polarizing factor IL-12p70 in response to LPS and CD40 ligand triggering. DC exposed to GA induce effector IL-4-secreting Th2 cells and enhanced levels of the anti-inflammatory cytokine IL-10. The anti-inflammatory effect of GA is mediated via DC as GA does not affect the polarization patterns of naive Th cells activated in an APC-free system. Together, these results reveal that APC are essential for the GA-mediated shift in the Th cell profiles and indicate that DC are a prime target for the immunomodulatory effects of GA. *The Journal of Immunology,* 2003, 170: 4483–4488.

Dendritic cells (DC)[3] make up a family of highly specialized cells that upon activation by pathogens or their products mature into professional APC. Immature sentinel DC sample peripheral tissues (e.g., skin, lung, and gut epithelia) for incoming pathogen-derived Ags. Thus activated, they mature and migrate through the lymphatics toward secondary lymphoid organs. During maturation, DC lose their capacity to internalize and process Ags, but up-regulate their costimulatory molecules (e.g., CD80, CD86, and CD40) to become potent immunogenic APC for naive CD4^{+} Th cells (1). In the T cell areas of lymphoid organs, mature effector DC not only present pathogen-derived peptides to T cells, but also adapt the class of immune response to the type of invading pathogen by driving the development of protective effector Th cell subsets. For instance, DC that have been activated by intracellular pathogens or their compounds, commit CD4^{+} Th cells to become protective IFN-γ-producing effector Th1 cells. Alternatively, helminths or certain extracellular pathogens induce DC that drive the development of protective IL-4-, IL-5-, and IL-13-producing effector Th2 cells. Whereas chronic activation of Th1 cells can cause immunopathology and organ-specific autoimmune disease, Th2 cells can mediate allergic and atopic disease (2–5).

An important DC-derived factor driving Th1 responses is bioactive IL-12p70 (2, 3). IL-12p70 can be secreted by sentinel DC in response to several pathogens (bacteria, viruses) or to pathogen-derived products (LPS, DNA, dsRNA) (6–10). However, during the priming of naive Th cells in the lymphoid organs, the secretion of IL-12p70 is induced upon interaction between CD40 on the DC and the rapidly induced CD40 ligand (CD40L, CD154) on the activated Th cell (6, 9, 11, 12). The levels of IL-12p70 production upon this CD40L-dependent activation are subject to regulation, reflecting the type of pathogen that activated the DC at its sentinel stage in the peripheral tissue concerned (4, 5). We and others have shown that in addition to the type of pathogen, immune mediators and drugs may also determine the levels of IL-12 produced by CD40L-activated mature DC. For instance, the immune mediators PGE₂, histamine, and IL-10, and the anti-inflammatory drugs, glucocorticoids, and β₂-agonists all prime DC for reduced IL-12p70 production upon CD40L-dependent activation, thus resulting in DC with enhanced Th2-promoting capacity (13–19). This has important implications for the design of therapeutic strategies aimed at counteracting Th1-associated immune pathologies.

Glatiramer acetate (GA), also known as copolymer-1, Cop-1, and Copaxone, is a synthetic random polymer of the amino acids L-alanine, L-glutamate, L-lysine, and L-tyrosine (20). GA was shown to suppress the induction of experimental autoimmune encephalomyelitis (EAE) in response to the encephalitogenic Ags myelin basic protein (MBP), proteolipid protein (21), and myelin oligodendrocyte glycoprotein (20–22). More recently, GA was also shown to inhibit type II collagen-reactive T cells in vitro (23) and to prevent graft vs host disease (24, 25) and transplant rejection (26). The potential of GA as a therapeutic agent in multiple sclerosis (MS) has been further substantiated by the reduction of relapse frequency in relapsing-remitting MS patients and by the reduced appearance of new lesions in gadolinium-enhanced magnetic resonance imaging (27–30).

It is generally accepted that the therapeutic effects of GA are due to its immunomodulatory effects on T cells (for a review, see Ref.

31). GA has been shown to bind promiscuously to class II MHC molecules in vitro (32–34), thus competing with MBP-derived peptides for the MBP-specific TCR (35–37). Interestingly, GA induces a Th1 to Th2 shift accompanied by the production of the anti-inflammatory cytokine IL-10 both in vitro and in vivo (34, 38–44). Hitherto, the actual mechanism underlying the induction of these protective Th cell responses remains elusive. Because the cytokine profile of Th cells is often regulated by DC, we have studied the modulatory effects of GA on the T cell regulatory function of human DC.

In this study we show that GA exerts its anti-inflammatory action on DC by potently inhibiting the production of the major Th1-polarizing factor IL-12p70 and of the proinflammatory cytokines TNF-α and IL-8 in response to inflammatory signals. Moreover, although GA does not phenotypically affect the maturation of DC, exposure of DC to GA during their maturation results in a stably reduced IL-12p70-producing capacity in response to subsequent activation by the T cell-derived signal CD40L. This results in the polarization of precursor naive Th cells into effector IL-4-producing Th2 cells and is accompanied by enhanced secretion of the anti-inflammatory cytokine IL-10.

Materials and Methods
Reagents

Human rGM-CSF (sp. act. 1.11×10^7 U/mg) was a gift of Schering-Plough (Uden, The Netherlands). Human rIFN-γ (sp. act. 8×10^7 U/mg) was a gift of Dr. P. H. van der Meide (U-CyTech, Utrecht, The Netherlands). Human rIL-2 was obtained from Cetus (Emeryville, CA). Human rIL-4 (sp. act. 1×10^8 U/mg) was obtained from Pharma Biotechnologie Hannover (Hannover, Germany). Escherichia coli-derived LPS was obtained from Difco (Detroit, MI) and was used at an optimal concentration of 100 ng/ml. Superantigen Staphylococcus aureus enterotoxin B (SEB) was obtained from Sigma-Aldrich (St. Louis, MO) and was used at a final concentration of either 10 or 100 pg/ml as indicated in Figs. 3 and 4. Water-soluble GA (COP-1; Sigma-Aldrich) was used at the final concentrations of 1, 3, and 10 μg/ml based on pilot experiments. Mouse anti-human CD3 mAb (CLB-T3/3) and mouse anti-human CD28 mAb (CLB-CD28/1) were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands) and were used at the final concentrations of 1 and 2 μg/ml, respectively, unless indicated otherwise (Fig. 5).

Generation of immature DC from peripheral blood monocytes, induction of DC cytokine production, and induction of DC maturation

Immature CD1a$^+$CD14$^-$ DC were generated from peripheral blood monocytes cultured in IMDM (Life Technologies, Paisley, U.K.) containing gentamicin (86 μg/ml; Duchefa, Haarlem, The Netherlands), 1% FCS (HyClone Laboratories, Logan, UT), GM-CSF (500 U/ml), and IL-4 (250 U/ml) (6). At day 6, immature DC (2×10^4 cells/well) were stimulated with LPS in either the absence or the presence of IFN-γ (10^3 U/ml) in 96-well flat-bottom culture plates (Costar, Cambridge, MA) in IMDM containing 10% FCS in a final volume of 200 μl. GA (1, 3, or 10 μg/ml) was added either alone or simultaneously with LPS $\pm$ IFN-γ. Supernatants were harvested after 20 h. Alternatively, at day 6, immature DC were induced to mature by a 2-day exposure to LPS (100 ng/ml) in the absence or in the additional presence of GA (1, 3, or 10 μg/ml). All subsequent tests were performed after harvesting the cells at day 8 and after extensive washing (four times in 10 ml of culture medium) to remove residual factors. Mature DC (2×10^4 cells/well) were stimulated with the CD40L-transfected J558 cell line (2×10^4 cells; a gift of Dr. P. Lane, University of Birmingham, Birmingham, U.K.) in 96-well flat-bottom culture plates (Costar) in IMDM containing 10% FCS in a final volume of 200 μl. Supernatants were harvested after 20 h. The levels of IL-12p70, IL-6, IL-8, IL-10, and TNF-α were measured by ELISA (see below).

Isolation of naive CD4$^+$CD45RA$^+$CD45RO$^-$ Th cells

Naive CD4$^+$CD45RA$^+$CD45RO$^-$ Th cells were isolated from peripheral blood leukocytes, with the negative selection human CD4$^+$CD45RO$^-$ column kit (R&D Systems, Minneapolis, MN). This method yielded highly purified naive Th cells as assessed by flow cytometry (>98%

CD4$^+$CD45RA$^+$CD45RO$^-$, <1% CD14$^+$, <1% CD20$^+$, data not shown).

APC-dependent and -independent priming of cytokine production in maturing Th cells

Naive Th cells (2×10^4 cells) were cocultured in 96-well flat-bottom culture plates (Costar) in IMDM containing 10% FCS with allogeneic mature DC (5×10^3 cells) or with autologous mature DC (5×10^3 cells) in the presence of SEB (10 pg/ml or 100 pg/ml) in a final volume of 200 μl. Before the coculture, DC were induced to mature by exposure to LPS in either the absence or the presence of GA (1, 3, or 10 μg/ml). Alternatively, naive Th cells (1×10^5 cells/200 μl) were stimulated in the absence of DC with soluble anti-CD3 and anti-CD28 mAbs in the absence or the presence of GA (1, 3, or 10 μg/ml) in 96-well flat-bottom culture plates in IMDM containing 10% FCS. Both in the APC-dependent and in the APC-independent activation protocols, IL-2 (10 U/ml) was added on day 5, and the cultures were further expanded for another 9 days. On day 14, resting memory Th cells were harvested, washed, and restimulated with PMA (Sigma-Aldrich) and ionomycin (Sigma-Aldrich) in the presence of brefeldin A (Sigma-Aldrich) to detect the intracellular production of IL-4 and IFN-γ. Alternatively, at day 14 resting Th cells were restimulated with soluble anti-CD3 and anti-CD28 mAbs (13), and the concentrations of IL-10 in 72-h supernatants were determined by ELISA (see below).

Induction and measurement of proliferative response in naive Th cells

Naive Th cells (2.5×10^4 cells/200 μl) were cocultured in 96-well flat-bottom culture plates (Costar) with increasing numbers of allogeneic DC (10^3–10^4) or with autologous DC in the additional presence of 100 pg/ml SEB. Cell proliferation was assessed by measuring the incorporation of [^{3}H]TdR (Radiochemical Center, Amersham Pharmacia, Little Chalfont, U.K.) by liquid scintillation spectroscopy after a pulse with 13 KBq/well during the last 16 h of a 3-day (SEB) or 7-day (MLR) culture.

Evaluation of cytokine production at the single-cell level

To evaluate intracellular cytokine expression, day 14 resting Th cells were harvested, washed, and restimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 6 h, the last 5 h in the additional presence of brefeldin A (10 μg/ml). Thereafter, the cells were washed in PBS, fixed for 15 min at room temperature in PBS containing 2% paraformaldehyde, and stained in permeabilization buffer (PBS containing 0.5% saponin, 1% BSA, and 0.05% NaN$_3$) with FITC-labeled mouse anti-human IFN-γ (IgG2b) and PE-labeled mouse anti-human IL-4 (IgG1) or the respective isotype-matched controls (all from BD PharMingen, San Diego, CA). Subsequently, the cells were washed, suspended in PBS containing 1% BSA and 0.05% NaN$_3$, and analyzed by flow cytometry. Data were analyzed using WinMDI software (http://facs.scripps.edu/).

Evaluation of cytokine production by ELISA

Determination of IL-12p70 concentrations in culture supernatants was performed by specific solid-phase sandwich ELISA as described previously (6). Pairs of specific mAbs and recombinant cytokine standards were obtained from BioSource International (Camarillo, CA) for the determination of IL-6, IL-8, and TNF-α, and from BD PharMingen for the determination of IL-10. The detection limits of these ELISA are as follows: IL-6, 20 pg/ml; IL-8, 30 pg/ml; IL-10, 25 pg/ml; IL-12p70, 3 pg/ml; and TNF-α, 20 pg/ml.

Statistical analysis

Data were analyzed for statistical significance with the GraphPad InStat software (version 3.00; GraphPad, San Diego, CA) using ANOVA followed by Dunnett's multiple comparison test. A p value of <0.05 was considered to be the level of significance.

Results
GA is a selective inhibitor of cytokine production by tissue-type sentinel DC

Because GA is therapeutically applied s.c., we first addressed the question of whether GA influences the sentinel function of tissue-type DC. To this end, immature DC were activated by exposure to LPS either in the absence or in the presence of increasing doses of GA. Fig. 1 depicts the regulatory effect of GA on the production of several DC-derived inflammatory mediators. The secretion of the
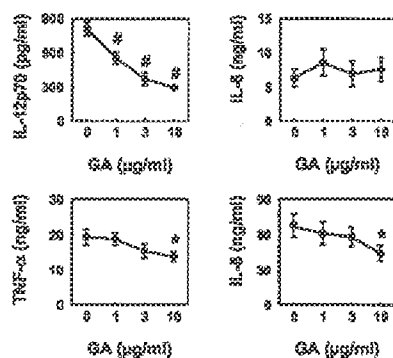
FIGURE 1. GA is a selective inhibitor of cytokine production by immature DC. Day 6 immature DC were stimulated with LPS in the absence or the presence of the indicated concentrations of GA. The concentrations of IL-12p70 (induced in the presence of IFN-γ), IL-6, IL-8, and TNF-α in 20-h supernatants were evaluated by ELISA. Results, expressed as the mean ± SD of triplicate cultures, are from one experiment representative of six. Data were analyzed for statistical significance using ANOVA followed by Dunnett's multiple comparisons test. *, $p < 0.05$; #, $p < 0.01$.

Th1-polarizing cytokine IL-12p70 was clearly inhibited by the presence of GA in a dose-dependent manner. Similarly, although to a lesser extent, GA reduced the secretion of the chemoattractant IL-8 and of the proinflammatory cytokine TNF-α. In contrast, IL-6 secretion was hardly affected in the range of GA doses tested, suggesting a selective role for GA in the regulation of DC cytokine production. The production of IL-10 by the DC was not detectable in any condition. In the absence of LPS, GA by itself did not induce DC cytokine production (data not shown).

Effector DC matured in the presence of GA have a reduced IL-12p70-producing capacity, but not an impaired immunostimulatory capacity

We and others have shown previously that certain immune mediators and anti-inflammatory drugs that inhibit IL-12p70 production in sentinel DC also prime maturing DC for a stable effector phenotype with a reduced capacity to secrete IL-12p70 in response to subsequent CD40L-dependent activation (13–19). To study whether GA exerts a similar effect, DC were matured by exposure to LPS during 48 h in the absence or the presence of increasing doses of GA. Subsequently, DC were thoroughly washed to remove residual factors, and stimulated with CD40L-transfected J558 cells. Fig. 2 shows that maturation of DC in the presence of GA resulted in a dose-dependent reduction of their capacity to secrete IL-12p70 in response to subsequent activation by CD40L. A comparable effect was observed for TNF-α, whereas IL-8 secretion was inhibited to a much lesser extent. In contrast, IL-6 production was hardly affected, again suggesting that GA selectively regulates DC function. The production of IL-10 by the DC was not detectable in any condition. Similar results were obtained when DC maturation was induced by the combination of IL-1β and TNF-α instead of LPS (data not shown), suggesting that the effect of GA does not critically depend on microenvironmental conditions.

Maturation-associated phenotypical changes are characterized by the up-regulation of class II MHC, the costimulatory molecules CD40, CD80, CD86, OX40L, ICAM-1, and the acquisition of the mature DC marker CD83. DC matured with LPS (or with IL-1β/TNF-α) acquired these mature phenotype markers irrespective of the presence of GA (data not shown). Mature DC are the exclusive effector APC for lymph node-based naive Th cells and conse-
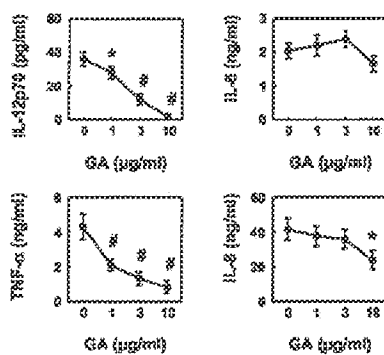

FIGURE 2. DC exposed to GA during their maturation have a reduced IL-12p70-producing capacity upon subsequent CD40L-dependent activation. Day 6 immature DC were induced to mature by a 2-day exposure to LPS in the absence or the presence of the indicated concentrations of GA. After 48 h, at day 8, matured DC were washed thoroughly to remove residual factors. DC were then stimulated with the CD40L-transfected J558 cell line, and 20-h supernatants were collected for cytokine measurement by ELISA. Results, expressed as the mean cytokine concentration ± SD of triplicate cultures, are from 1 experiment representative of 10. Data were analyzed for statistical significance using ANOVA followed by Dunnett's multiple comparisons test. *, $p < 0.05$; #, $p < 0.01$.

quently for the initiation of specific immune responses (1). Therefore, we addressed the question of whether exposure of maturing DC to GA affects their immunostimulatory capacity toward naive Th cells, by testing the capacity of GA-treated DC to induce proliferation of either allogeneic naive Th cells or autologous naive Th cells in response to SEB. Fig. 3 shows that exposure of DC to GA during maturation did not impair their immunostimulatory potential as judged by the similar proliferation rates of naive Th cells in response to DC exposed to increasing doses of GA both in the MLR and in the SEB models. These results are in line with the unaffected acquisition of the mature phenotype. Together, these data suggest that in the presence of GA, DC become genuine effector APC with a stably reduced capacity to produce IL-12p70.
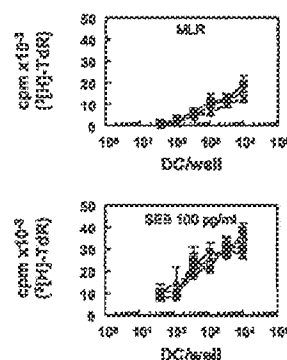

FIGURE 3. DC exposed to GA are not impaired in their capacity to activate naive Th cell proliferation. DC were matured with LPS in the absence (O) or the presence of increasing doses of GA (1 μg/ml, ●; 3 μg/ml, ■; or 10 μg/ml, ▲). After extensive washing, increasing numbers of DC were used to stimulate either 2.5×10^4 allogeneic naive Th cells (MLR) or 2.5×10^4 autologous naive Th cells in the presence of 100 pg/ml SEB. The proliferative response was determined either at day 3 (SEB) or day 7 (MLR) of coculture by [^{3}H]TdR incorporation. Results, expressed as the mean cpm ± SD of triplicate cultures, are from one experiment representative of four.

DC matured in the presence of GA induce Th2 cells accompanied by high levels of IL-10

The levels of DC-derived IL-12p70 play a major role in Th1 polarization (2, 3). Hence, we studied to what extent the exposure of DC to GA hampers the early commitment of naive Th cells to become Th1 cells. To this aim, effector DC were generated by maturation with LPS in the absence or the presence of increasing doses of GA. After 48 h the cells were thoroughly washed and used to stimulate allogeneic naive Th cells or autologous naive Th cells in the presence of SEB. The polarization of effector Th cells was evaluated by determining the production ratios of signature Th1 (IFN-γ) to Th2 (IL-4) cytokines at the single-cell level. Fig. 4*A* shows that DC that were matured in the presence of increasing doses of GA promoted the development of IL-4-producing effector Th cells and inhibited the generation of IFN-γ-producing Th cells. The dose-dependent shift in the ratio of IFN-γ-producing cells to IL-4-producing cells positively correlated with the dose-dependent inhibitory effect of GA on the IL-12p70-producing capacity of DC and indicates that GA modulates DC-derived IL-12p70 to control the polarization profiles of inflammatory Th cells. We subse-
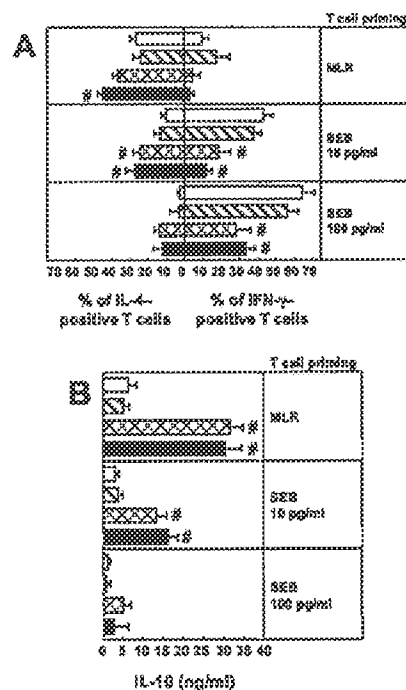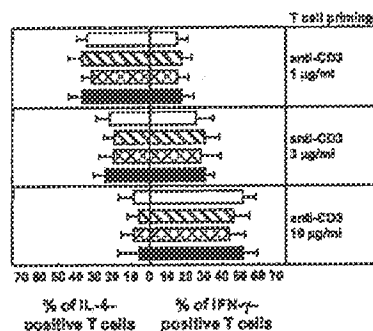

FIGURE 5. GA does not affect naive Th cell polarization in the absence of APC. Naive Th cells were activated with the indicated concentrations of soluble anti-CD3 and soluble anti-CD28 mAbs in the absence (☐) or the presence of increasing doses of GA (1 μg/ml, ▓; 3 μg/ml, ▓; 10 μg/ml, ▓). The cells were allowed to expand until they reached the resting state. On day 14, the quiescent Th cells were restimulated and analyzed for intracellular IL-4 and IFN-γ production as described in the legend to Fig. 4*A*. Results are the mean percentage of positive cells ± SD of three independent experiments.

quently evaluated whether this Th2 shift is accompanied by the induction of IL-10-producing Th cells. Fig. 4*B* shows that exposure of DC to GA during maturation resulted in the generation of Th cells with a strongly enhanced capacity to secrete the anti-inflammatory cytokine IL-10. Together, these results indicate that GA modulates the APC function of DC to induce anti-inflammatory Th cells producing IL-4 and/or IL-10.

To further investigate to what extent this anti-inflammatory effect of GA depends on the presence of APC, naive Th cells were activated in an APC-free system with soluble mAbs directed against CD3 and CD28 in the absence or the presence of increasing doses of GA. The obtained resting effector Th cells were restimulated and evaluated for their production of IFN-γ and IL-4 at the single cell level by flow cytometry. Fig. 5 shows that in the absence of APC, GA was unable to affect the balance between Th1 and Th2 cells, indicating that DC are critical for the GA-mediated shift in the Th cell profile.

Discussion

The present study shows for the first time that GA induces anti-inflammatory Th cell responses by modulating the APC function of DC and not by direct effects on the Th cells. GA selectively inhibits the production of DC-derived inflammatory mediators without affecting DC maturation and the DC immunostimulatory potential. DC exposed to GA during maturation have an impaired and stable capacity to secrete the major Th1-polarizing cytokine IL-12p70, resulting in the induction of a population with an increased frequency of effector IL-4-secreting Th2 cells accompanied by enhanced secretion of the anti-inflammatory cytokine IL-10.

It is generally accepted that GA exerts its effects by modulating T cell function (reviewed in Ref. 31). With respect to the binding of GA to immune cells, in vitro experiments have shown that GA binds promiscuously to class II MHC (32-34), thereby competing with MBP-derived peptides for the MBP-specific TCR (35-37). This is an implausible setting in vivo. GA is rapidly degraded into small peptides (43), and it is unlikely that 20 mg of GA administered s.c. would be capable of exerting TCR antagonism by displacing relevant auto-Ags in both the CNS and the immune system (46). GA is well tolerated, and s.c. application seldom results in

FIGURE 4. DC exposed to GA during maturation induce the development of IL-4-secreting Th2 cells with elevated IL-10-secreting capacity. Day 6 immature DC were induced to mature by a 2-day exposure to LPS in the absence (☐) or the presence of increasing doses of GA (1 μg/ml, ▓; 3 μg/ml, ▓; 10 μg/ml, ▓) and were used to stimulate allogeneic naive Th cells (MLR) or autologous naive Th cells with SEB. *A*, On day 14 the quiescent Th cells were restimulated with PMA and ionomycin for 6 h, the last 5 h in the presence of brefeldin A, to detect the intracellular production of IL-4 and IFN-γ. Results are the mean percentage of positive cells ± SD of three independent experiments representative of seven. *B*, Alternatively, the quiescent Th cells were restimulated with anti-CD3 and anti-CD28 mAbs, and the concentrations of secreted IL-10 in 72-h supernatants were determined by ELISA. Results, expressed as the mean IL-10 concentration ± SD of triplicate cultures, are from one experiment representative of five. Data were analyzed for statistical significance using ANOVA followed by Dunnett's multiple comparisons test. #, *p* < 0.01.

skin reactions (28). However, there are indications of immune activation in vivo that suggest the active participation of APC in mediating the effects of GA. A recent study reported lymphadenopathy in 30% of MS patients following GA administration (47). Moreover, a recent report supports our observations that the anti-inflammatory effects of GA are mediated via the APC. Wiesemann et al. (48) showed that GA induces IL-5 and IL-13 production in naive Th cells only if the cells are activated in the presence of CD14+ cells. This effect is dependent on Ag presentation as demonstrated by TCR blocking Abs. It is noteworthy that GA induces a Th1 to Th2 shift accompanied by the production of the anti-inflammatory cytokine IL-10 both in vitro and in vivo (34, 38–44). It is well established that the cytokine profile of Th cells is often regulated by DC. Hitherto, there is no information regarding the effects of GA on the effector function of DC. Very recently, Hussien et al. (49) reported that the presence of GA during the in vitro generation of immature DC from human peripheral blood monocytes inhibited the capacity of these immature DC to produce biologically inactive IL-12p40. This study left the question whether GA could directly modulate the production of bioactive IL-12p70 of immature DC or could modulate the maturation of these DC and consequently their capacity to induce Th1 responses unanswered. The data reported in this study show for the first time that DC are critically involved in the mechanisms underlying the immunomodulatory effects of GA on Th cells. In addition to the direct inhibitory effect of GA on the secretion of inflammatory cytokines, in particular IL-12p70 production by immature DC in response to LPS, GA primes maturing DC for deficient IL-12p70 production in response to subsequent CD40 triggering. The levels of IL-12p70 secreted by the DC are of key importance in determining the class of the primary immune response (2, 3). By inhibiting the IL-12p70-producing capacity of DC, GA suppresses the development of Th1-associated profiles. The immunostimulatory potential of DC primed by GA is not compromised, probably because GA does not affect their maturation status. In this study we demonstrate that the Th cells that develop in response to the GA-primed DC secrete enhanced levels of IL-10. It is well established that at low IL-12p70 levels, autocrine IL-4 production boosts the development of Th2 cells (2, 3). In contrast, the nature of the signal(s) driving the induction of high-level IL-10-secreting cells in low IL-12p70 conditions is unknown. In experiments using supernatants of activated DC we observed that GA-modulated DC induce Th cell polarization via as yet unknown soluble factor(s) (data not shown). Moreover, in experiments using neutralizing anti-IL-10 mAb during the activation of naive Th cells by these DC, the acquisition of the high-level IL-10-producing capacity was not prevented (data not shown). The putative role for other candidate molecules responsible for the induction of high-level IL-10-producing T cells, such as the glucocorticoid-induced TNFR family-related protein and its ligand (50), needs further investigation.

The actual molecular mechanisms by which GA affects the production of immune mediators by DC and consequently directs the generation of anti-inflammatory Th cells remains elusive. Recently, it was described that GA inhibits the IL-1-dependent activation of NF-κB in astroglial cells (51). Indeed, the NF-κB family of transcription factors is involved in the regulation of activation of DC and of cytokine production in myelomonocytic cells (1, 52, 53). In this respect, NF-κB family member RelA and IκB (53) may be targets for the GA regulatory effect. We have preliminary evidence suggesting that activation of DC in the presence of GA (1–10 μg/ml) results in reduced translocation of RelA to the nucleus with retention of RelA in the cytoplasmic compartment (our unpublished results). Whether this effect of GA on RelA is medi-

ated through IκB is not yet clear. These issues are currently under investigation.

We have shown in this study that DC are pivotal in the transmission of protective Th2/IL-10 responses, a mechanism that has not been described before to explain the anti-inflammatory effects of GA. GA-primed DC will drive the development of anti-inflammatory effector Th cells from naive T cells responding to any Ag presented by these DC. The notion that this effect of GA on DC is Ag-nonspecific may explain that GA is not only effective in EAE (34, 38, 39) but also in graft vs host disease (25) and transplant rejection (26) models as well as in MS patients (40–44). At the same time, however, this finding does not exclude the fact that this polypeptide has additional Ag-specific effects. In the specific case of EAE and MS, GA-primed DC may activate GA-specific Th cells that will further compete with, e.g., MBP-specific Th cells for binding to MBP peptides presented in the context of class II MHC. In contrast, APC loaded with GA may also compete with APC loaded with MBP peptides for MBP-specific TCR, and may help to shift the cytokine profiles of these inflammatory MBP-specific T cells.

Acknowledgments

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Exhibit F
Zhang, *et al.* (2002)
"Mature bone marrow-derived dendritic cells polarize Th2 response and suppress experimental autoimmune encephalomyelitis."
Multiple Sclerosis 8: 463–468

Mature bone marrow-derived dendritic cells polarize Th2 response and suppress experimental autoimmune encephalomyelitis

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Distinct subsets of dendritic cells (DCs) based on the origin, phenotypes, and the nature of the signals that promote DC maturation can determine polarized immune responses of T cells. In this study, DCs were cultured from mouse bone marrow (BM) progenitors in granulocyte–macrophage colony-stimulating factor (GM-CSF). To generate mature DCs (mDCs), lipopolysaccharide (LPS) was used in the culture for 24 h. LPS-stimulated DCs were phenotypically mature, which exhibited strongly upregulated CD40, B7.1, and B7.2 compared to non-LPS-stimulated immature DCs (imDCs). Both mDCs and imDCs expressed high levels of MHC class II but low level of CD54. mDCs produced higher levels of IL-10 and lower IL-12 compared to imDCs. No IFN-γ or IL-4 was found in both groups. When mDCs were injected intraperitoneally (i.p.) to the mice with experimental autoimmune encephalomyelitis (EAE), the severity of clinical signs and inflammation in the CNS was significantly suppressed compared to imDC-injected mice ($p < 0.01$) and PBS-injected mice ($p < 0.02$). Moreover, lymphocytes from mDC-injected mice produced lower level of IL-12, IFN-γ, but higher level of IL-10, compared to imDC-injected and non-DC-injected mice. We conclude that BM-mDCs, but not BM-imDCs, promote Th2 differentiation and have the potential for suppression of inflammatory demyelination.
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Key words: cytokines; dendritic cells; experimental autoimmune encephalomyelitis; multiple sclerosis; Th1/Th2

Introduction

Specific immune responses are orchestrated by type 1 and 2 cytokines produced by antigen-activated Th cells.[1] However, less is understood about the cellular and molecular basis of Th1/Th2 development exerted by other parameters, such as how antigen-presenting cells (APCs) influence this process. Experimental autoimmune encephalomyelitis (EAE), an animal model for the human disease multiple sclerosis (MS), is a T-cell-mediated central nervous system (CNS) autoimmune disease. The autoreactive T cells, directed against neuroantigens, including myelin basic protein and myelin oligodendrocyte glycoprotein (MOG), are of the Th1 type that produces IFN-γ and TNF and promotes cell-mediated immunity, while Th2 type cells that produce IL-4 and IL-10 contribute to the disease remission and tolerance induction.[2] IL-12, an important cytokine produced by activated APCs, induces IFN-γ production by T and NK cells and is pivotal in Th1-type immune response development.[3] IL-12 is involved in the induction and maintenance of EAE.[3-7] Understanding the cellular and molecular events responsible for the preferential polarization toward Th1 or Th2 responses at APC stage could help identify new targets of therapeutic intervention in EAE and MS.

Dendritic cells (DCs) are the most potent APCs that possess the capacity to activate naïve T cells to initiate immune response.[8,9] DCs were previously considered as a distinct lineage of antigen-presenting leukocytes with potent capacity to induce primary T-cell-mediated immune responses.[10] Recently, it has been proposed that DCs should be regarded as a multiple-lineage system of leukocytes with variable functions rather than a homogenous cell type with predetermined functional properties.[10] Two possibilities could result in this multilineage and multifunctional: different subsets of DCs, or the activation/maturation state of DCs regardless of their origin or subtype. These differences could decide DCs as the initiator and modulator of the immune response.[11] Several studies have shown that certain DC populations could be immunoregulatory and able to suppress diabetes,[11-14] EAE[15-17] and extend the survival of transplantation.[18,19] In vitro antigen-pulsed bone marrow-derived DCs[14] and DCs from EAE rats[15] could induce tolerance against EAE. The effect of DC maturation on the pathogenesis of EAE has not been addressed. In the present study, we investigate the effect of maturation state of bone marrow-derived DCs on the induction and maintenance of MOG-induced EAE. Th1/Th2 polarization driven by immature (imDCs) and mature DCs (mDCs) was further studied.

Materials and methods

Generation of DCs from bone marrow

DCs from femurs and tibiae of female, seven- to eight-week old C57BL/6J mice were prepared as previously described.[20] Briefly, bone marrow (BM) cells were flushed with PBS

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using a syringe with a 0.45 mm-diameter needle. After clusters within the marrow suspension were disintegrated by vigorous pipetting and washing in PBS, the suspension of leukocytes were cultured in bacteriological 100 mm Petri dishes (Falcon, No. 1029/Becton Dickinson, Heidelberg, Germany) at 2×10^6 per dish in 10 ml culture medium. Cell culture medium was RPMI-1640 (Gibco-BRL, Eggenstein, Germany) supplemented with penicillin (100 U/ml, Sigma, St. Louis, MO), Streptomycin (100 μg/ml, Sigma), L-glutamine (2 mM, Sigma), 2-mercaptoethanol (50 μM, Sigma), 10% heat-inactivated and filtered (0.22 μm, Millipore Inc., Bedford, MA) FCS (Sigma), and 20 ng/ml of recombinant murine granulocyte–macrophage colony-stimulating factor (GM-CSF, PeproTech, Rocky Hill, NJ). At day 3, another 10-ml culture containing 20 ng/ml GM-CSF was added to the plates. At day 6, half of the culture supernatant was collected, centrifuged, and the cell pellets resuspended in 10 ml fresh culture medium containing 20 ng/ml of GM-CSF, and returned to the original plate. At day 8, the nonadherent cells were collected by gentle pipetting and were seeded into new dishes. Ten milliliters supernatant each dish was replaced by fresh culture medium containing 20 ng/ml GM-CSF. For complete maturation, 1 μg/ml of lipopolysaccharide (LPS, Sigma) was supplemented into the culture at day 9 (mDCs), and the wells without LPS served as imDCs. At day 10, cells were collected by gentle pipetting, washed in PBS, counted, and were ready to use. Supernatants were collected and stored at −70°C for future ELISA assays. With 10 ng/ml of GM-CSF these cells could be kept to day 13 for use. One microgram LPS was provided at day 12 to imDC plates to certain dishes to yield mDCs.

Flow cytometry of purified DCs
The purity of DCs and the maturation status were determined by flow cytometry. The following antibodies were used in this study: CD11c (HL3, hamster IgG), I-A/I-E (2G9, rat IgG2a), CD80 (B7-1, 16-10A1, hamster IgG), CD86 (B-7.2, GL1, rat IgG2a), CD54 (ICAM-1, 3E2, hamster IgG), CD40 (3/23, rat IgG2b), CD3 (145-2C11, hamster IgG), B220 (RA3-6B2, rat IgG2a), and isotype controls.

Cytokine production by DCs
Supernatants collected from mDC and imDC cultures were analysed with anti-mouse IFN-γ, IL-4, IL-10, and IL-12 p70 (all from Pharmingen, San Diego, CA) antibody pairs following the manufacturer's instructions. The concentrations of detected samples were calculated based on the standard curves of known concentrations of relative cytokines.

Induction of EAE
Mice were each injected subcutaneously with 400 μg MOG peptide 35–55 (MOG35–55) in complete Freund's adjuvant (CFA) containing 4 mg/ml *Mycobacterium tuberculosis* over two sites at the back. Pertussis toxin (500 ng; List Biological Laboratories, Campbell, CA) was given intraperitoneally (i.p.) on days 0 and 2 post immunization (p.i.). Peptide was synthesized and purified with HPLC by the Protein Chemistry Laboratory of the University of Pennsylvania School of Medicine supported by core grants of the Diabetes and Cancer Centers (DK-19525 and CA-16520).

In vivo treatment with DCs
At day 0 p.i., 2×10^6 mDCs and imDCs were suspended into 0.5 ml of PBS and administered i.p. to each mouse, every three days for five times. DCs were washed three times with PBS before injection. Mice receiving the same amount of PBS (non-DCs) served as controls ($n=8$ in each group). Clinical scores were determined daily by two blinded observers. A clinical scoring system with a scale of 0 to 5, with 0.5 points for intermediate signs, was used as follows: 0, normal; 1, flaccid tail, abnormal gait; 2, hind leg weakness or severe ataxia; 3, minimal hind leg movement; 4, hind leg and forelimb paralyzed; 5, moribund or dead.[21] All work was performed in accordance with the guidelines for animal use and care at the University of Pennsylvania.

Histopathological assessment
To determine pathological signs, animals were perfused through the left ventricle with 50 ml of physiological saline containing 2 U/ml heparin. Brains and spinal cords were harvested at day 28 p.i., embedded in paraffin, and sections were stained with haematoxylin and eosin for assessment of inflammation. Two investigators unaware of the experimental groups to which the tissues belonged, assessed inflammation as follows: 0, none; 1, a few inflammatory cells; 2, organization of perivascular infiltrates; and 3, increasing severity of perivascular cuffing with extension into the adjacent tissue.

Cytokine production of lymphocytes of DC-treated mice
Suspensions of mononuclear cells (MNCs) were prepared as described[22] from the popliteal and inguinal lymph nodes from imDC-, mDC-, and PBS-injected mice and cells were suspended in RPMI-1640 supplemented with 1% (vol/vol) MEM (Gibco), 2 mM glutamine (Life Technologies, Rockville, MD), 50 IU/ml penicillin, 50 mg/ml streptomycin, and 10% (vol/vol) FCS (Gibco). The cells were washed three times and then rediluted to a cell concentration of 2×10^6/ml. MNCs were cultured in the presence and absence of MOG35–55. After 48 h, supernatants were collected and the production of IFN-γ, IL-4, IL-10 and IL-12 were analysed by ELISA.

Statistics
Mann–Whitney U-test was employed for comparing clinical courses. ANOVA and Student's t-test were used to compare degrees of inflammation and demyelination. $p < 0.05$ was considered significant.

Results

Yield, purity, and maturation of DCs
DC yield, purity and maturation status was determined before these cells were injected. At day 10, nonadherent cells were harvested and approximately 50×10^6 of DCs were obtained per mouse. There was no difference in the number between LPS-stimulated and non-LPS dishes. By exchanging the supernatants with 10 ml fresh culture medium containing 20 ng/ml of GM-CSF, DCs could be kept
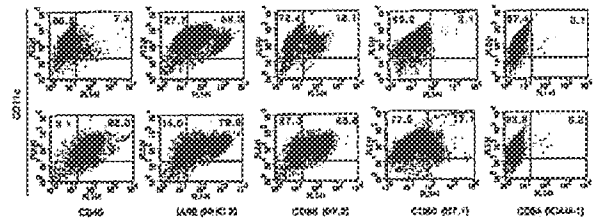
Figure 1 Cell surface markers of BM-DCs. On days 10 and 13 of culture, nonadherent cells were harvested and approximately 50×10⁶ DCs were obtained per mouse. There was no difference in the number of DCs in the LPS-stimulated and non-LPS dishes. DCs were defined as $CD11c^+CD3^-B220^-$ and their purity was 90–93%. BM-DCs (1×10⁶) were washed, stained with directly labeled antibodies and analysed with four-color FACScan. Data represent 10,000 events. Numbers represent the percentages of different populations. Similar results were obtained from five repeated experiments

until day 13 for use without affecting the viability. Purity of DCs was defined as $CD11c^+CD3^-B220^-$ and the percentage of these cells between day 10 to day 13 was 90–93%. Figure 1 showed flow cytometry results from day 10; similar results were obtained at day 13. The exposure of BM-derived DCs to LPS results in the initiation of their final maturation. This was evidenced by the upregulation of surface expression of CD40 (92% versus 7.4%), B7.1 (17.1% versus 2.1%), and B7.2 (65.6% versus 18.1%) compared to non-LPS-stimulated imDCs. Both mDCs and imDCs expressed low levels of CD54 (0.1% and 0.2%) and high levels of MHC class II (79.6% versus 69.3%).

Cytokine production of imDCs and mDCs

To further identify the phenotype of mDCs and imDCs, IFN-γ, IL-4, IL-10 and IL-12 production was measured from the culture supernatants. As shown in Figure 2, mDCs produced higher levels of IL-10 (7.1 ± 2.2 versus 3.5 ± 1.5 ng/ml;

$p < 0.05$) and lower levels of IL-12 (7.0 ± 0.5 vs. 14.0 ± 0.1 pg/ml; $p < 0.01$) than imDCs. No IFN-γ or IL-4 was detectable in both groups.

mDCs inhibit development of clinical EAE

We investigated the in vivo effect of imDCs and mDCs on clinical EAE (Figure 3). PBS-injected control mice exhibited characteristic signs of EAE starting on day 14 p.i. The maximal mean clinical score reached 3.5 ± 1.0, and then progressed. In imDC-injected mice, the maximal mean clinical score reached 4.3 ± 0.6. There was no significant difference between those two groups. Although mDC-injected mice exhibited similar onset as imDC-injected mice and non-DC-injected mice. These mice developed significantly less clinical scores of EAE. The maximal mean score was 2.5 ± 1.1, then gradually decreased to 1.2 ± 1 when these mice were sacrificed at day 28 p.i. The differences were significant between mDC-injected mice and PBS-injected
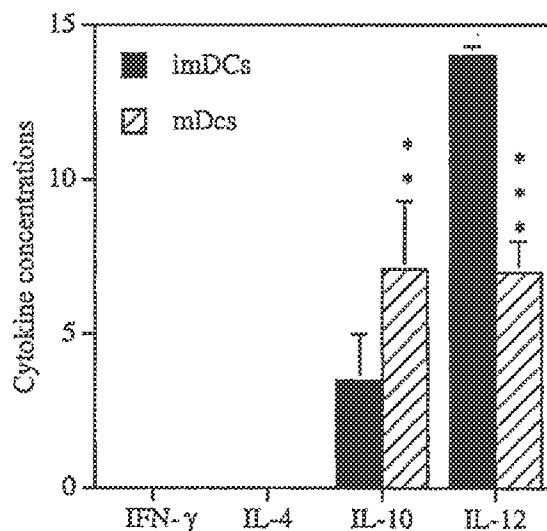

Figure 2 Cytokine production by imDCs and mDCs. Supernatants from imDCs and mDCs were collected and IFN-γ, IL-12, IL-4, and IL-10 production were tested in triplicates by sandwich ELISA. **$p < 0.01$, and *** $p < 0.001$. All experiments were repeated three times with similar results. Bars=SD
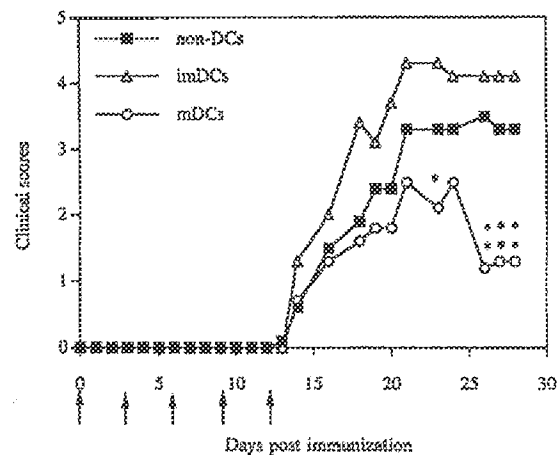

Figure 3 mDCs inhibit development of clinical EAE. At day 0 p.i., 2×10⁶ of either mDCs or imDCs were suspended into 0.5 ml of PBS and administered i.p. to each mouse, every three days for five times. Control mice were injected with PBS only (non-DCs). Clinical disease was evaluated daily in a masked manner. Symbols refer to mean clinical scores (n=4 in each group). (*) $p < 0.05$ and (**) $p < 0.02$ between mDC-injected mice and PBS-injected mice. Arrows (↓) refer to the days of treatment. The experiment was repeated (n=4 in each group) with similar results
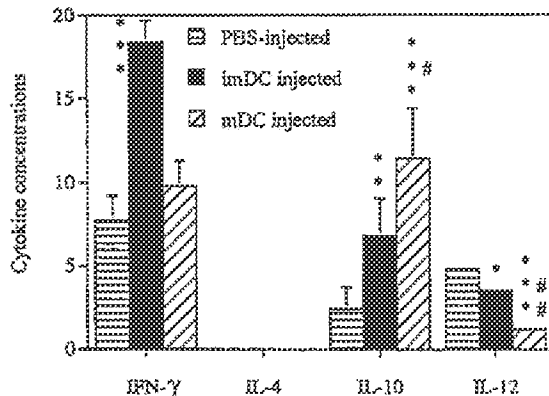
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Figure 4 MOG-induced cytokine production in DC-treated EAE mice. IFN-γ, IL-12, IL-4, and IL-10 production in DC-treated mice was detected in triplicate by sandwich ELISA. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ between PBS-injected mice and other groups. (#) $p < 0.05$ and (##) $p < 0.01$ between imDC- and mDC-injected mice. All experiments were repeated three times with similar results. Bars=SD

mice ($p < 0.02$). There was no significant difference between imDC-injected mice and PBS-injected mice.

Histology of EAE

CNS histology was assessed in mice that had been treated with imDCs, mDCs, and PBS. The infiltrates were dramatically suppressed in the spinal cord of mice injected with mDCs (1.0 ± 0.04) compared to imDC-injected mice (2.4 ± 0.2, $p < 0.01$) and PBS-injected control mice (2.1 ± 0.1; $p < 0.01$).

Cytokine production of mice treated with imDCs and mDCs

To study the immunoregulatory effect of DCs in EAE, we analysed the protein secretion of Th1/Th2 cytokines from the culture supernatant of lymphocytes of different groups. As shown in Figure 4, MNCs from mDC-injected mice produced lower level of IL-12 (1.2 ± 0.1 pg/ml) compared to PBS-injected mice (4.8 ± 0.1 pg/ml; $p < 0.001$) and imDC-injected mice (3.5 ± 0.1 pg/ml; $p < 0.01$) when stimulated with autoantigen. The difference in the level of IL-12 between imDC-injected mice and PBS-injected mice was also significant ($p < 0.05$). MOG-induced IFN-γ production was similar in mDC-injected mice (9.8 ± 1.5 ng/ml) and PBS-injected mice (7.7 ± 1.5 ng/ml) but higher in imDC-injected mice (18.4 ± 1.3 ng/ml) compared to PBS- or mmDC-injected mice ($p < 0.01$, respectively). Significantly higher level of IL-10 was found in mDC-injected mice (11.0 ± 3.0 ng/ml; $p < 0.001$) and imDC-injected mice (6.6 ± 2.2 ng/ml; $p < 0.01$) compared to PBS-injected mice (2.4 ± 1.3 ng/ml). Further, the level of IL-10 in mDC-injected mice was significantly higher than in imDC-injected mice ($p < 0.01$). No IL-4 was detectable in all three groups. These results suggest that the immunoregulatory property of mDCs is related to an elevated production of IL-10 and reduced IL-12.

Discussion

The present study provides evidence that BM-mDCs, but not BM-imDCs, promote Th2 differentiation and have the potential for suppression of clinical disease of EAE.

It has been suggested that APCs, among which DCs are the most potent, provided T cells not only with an antigen-specific stimulatory signal (signal 1, ligation of TCR) and a series of costimulatory signals (signal 2), but also with signals (proposed as signal 3) that polarize Th cell cytokine production.[1] There exists a paradigm of type 1 and type 2 DCs, i.e., DCs with high IL-12 production (DC1, promoting Th1 cell development) or DCs with no IL-12 production (DC2, promoting Th2 cell development).[13] Several factors might be responsible to the multiple functions of DCs. For example, two distinct lineages of human and mouse DCs have been described: myeloid DCs and lymphoid DCs.[24] In mice, lymphoid-derived DCs induce Th1 responses and myeloid DCs promote Th2 differentiation in vivo and in vitro,[25,26] while in the human system, lymphoid/plasmacytoid DCs were shown to generate Th2 responses and myeloid DCs to generate Th1 responses.[27] Even the ratio of stimulators (monocyte-derived DCs) and responders (CD4+ T cells) may affect the differentiation of naive T cells into different T-helper phenotypes.[28]

The state of maturation is suggested to play an important role in the determination of the resting T-cell activation and the type of T-cell response generated.[29] After stimulation with LPS or IL-4, imDCs could become mature. Cultures of BM cells in GM-CSF plus repeatedly high doses of LPS from day 0 generate imDCs that induce alloantigen-specific T-cell anergy in vitro, while LPS given from day 6 turn immature DCs into more mature DCs.[20] DCs are an important source of IL-12; however, DCs may secrete more IL-12 at earlier stages of maturation followed by a gradual decrease in IL-12 production at later stages of DC maturation, indicating that DCs may perform different functions at different stages of maturation.[28] In diabetes of NOD mice, both imDCs and IL-4-induced mDCs have the capacity to suppress spontaneous diabetes in those animals, while mDCs were optimal.[12] It was shown that MBP TCR transgenic mice transferred with Ac1-11-pulsed DCs developed EAE, whereas those mice receiving medium-pulsed DCs did not.[30] Interactions between antigen-pulsed DCs and antigen-specific T cells could be seen in the lymph nodes following adoptive transfer of both populations. These results indicated that DCs could effectively present self-antigen to antigen-specific T cells in the periphery of mice to induce EAE.[30] In Lewis rats, BM-derived DCs that have been pulsed in vitro with autoantigen peptide induced tolerance to actively induced EAE.[18] BM-DCs were also involved in IL-4-induced suppression of EAE by producing high levels of IFN-g, IL-10, and nitric oxide.[15] However, the effect of maturation of DCs on the development of EAE has not been studied. Our study showed that, upon stimulation with LPS, DCs exhibited strongly upregulated cell surface markers for maturation, namely CD86^{hi}, CD80^{hi}, and CD40^{hi}, compared to non-LPS stimulated imDCs. mDCs not only produced much less amounts of IL-12, consistent with the previous studies, but also produced much higher amounts of IL-10 compared to

imDCs. Although imDC-injected mice also had a tendency to induce less IL-12 and more IL-10 compared to non-DCs (Figure 4), this effect is not strong enough to overcome the pathogenesis. This strongly exhibited so-called DC2 phenotype[31] of mDCs, i.e., high level of IL-10 and low level of IL-12, therefore, potently polarized the immune system to Th2 responses in EAE.

How T cell develops into Th1 phenotype in autoimmune diseases remains unclear. One possible reason for T-cell autoreactivity in these diseases is the result of an inability of APCs to activate immunoregulatory T cells,[32] which leads to an imbalance between the effector and regulatory T-cell populations. IL-12 plays a crucial role in influencing the differentiation of T cells toward a Th1 phenotype.[33] IL-12 knockout mice were resistant to EAE in susceptible strains[34] and blocking IL-12 by antibodies confers an effective approach to abrogate clinical progression or relapse in EAE.[3,34] Our results suggest that the mDCs produced less IL-12 but more IL-10, therefore driving Th2 response after injection in MOG-induced EAE mice. The role of IFN-γ in the induction of EAE is controversial, which might have a proinflammatory or protective effect.[2,35–37] Although the primary effect of IL-12 is to upregulate the secretion of IFN-γ, in our study we found no correlation between the IL-12 levels and IFN-γ secretion in PBS-injected, imDC-injected, and mDC-injected mice, or between IFN-γ levels and clinical scores. Our data showed that elevated IL-12 in PBS-injected group promotes clinical EAE through a non-IFN-γ pathway.

In EAE, regulatory T cells are considered to play an important role in the resistance to EAE in some nonsusceptible strains.[38–40] Promoting the capacity of immunoregulatory APCs to drive Th2 polarization, could be a strategy to suppress Th1 cell-mediated autoimmune diseases like EAE. The factor(s) driving towards Th2 predominance is not yet defined. Lymphocytes of mDC-injected mice upon stimulation with MOG35–55 produced significantly higher level of IL-10 compared to PBS-injected and imDC-injected mice (Figure 4). These data indicate that mDC is a potent inducer of IL-10. IL-10 is produced by Th2 cells, NK cells, macrophages, and DCs. IL-10 downregulates costimulatory signals by macrophages,[33] DC-driven IFN-γ production by T cells, and T-cell responses to antigen through inhibition of IL-12 production and IL-12R chain expression.[41] DCs pretreated with IL-10 induce tolerance by converting imDCs into tolerogenic DCs.[42] Adoptive transfer of autoreactive T cells genetically designed to secrete IL-10 was able to delay the onset of EAE.[43] The observation that exposure of developing autoimmune effector cells to IL-10 hinders their development,[24] provides an explanation for our finding that the high level of IL-10 induced by administration after disease onset is potent to suppress ongoing EAE. Although no evidence for antigen-specific IL-4 production was observed in the treatment, we consistently observed antigen-specific IL-10 production. It has also be shown that TNF-α stimulated mature BM-DCs, but not imDCs, suppressed EAE by inducing IL-10.[44] Recent evidence showed that there is an IL-10/IL-12 immunoregulatory circuit controlling susceptibility to autoimmune disease.[34] In this circuit, the disease-promoting effects of IL-12 are antagonized by IL-10, which, in turn, is regulated by the exogenous production of IL-12. Therefore, manipulation of the IL-12/IL-10 balance could have profound effects on the incidence of autoimmune diseases.[24,45] Our study showed that mature BM-derived DC population possesses regulatory properties, as DC therapy has mediated the generation of Th2 bias and suppressed EAE by changing the balance between effector Th1 cells and regulatory Th2 cell populations in these mice. Upon injection, these cells may directly suppress the inflammation in EAE mice by producing IL-10 and/or by inducing other immunoregulatory cells, e.g., Th2 cells and Tr1 cells. The phenotype and the frequency of T cells derived from the protected and unprotected mice are subjects of ongoing studies in our laboratory.

In conclusion, our study demonstrates in vivo suppressive effect of mDCs on EAE, and suggests that mDCs selectively downregulate antigen-specific response through suppression of Th1 and induction of Th2 cytokines. In vitro manipulation of autogenous DCs to mature state could be a potential therapy for EAE and human MS in particular and autoimmune diseases in general.

Abbreviations

BM: bone marrow; CNS: central nervous system; DCs: dendritic cells; EAE: experimental autoimmune encephalomyelitis; MNC: mononuclear cells; MOG: myelin oligodendrocyte glycoprotein; p.i.: post immunization.

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